Volume 1 Number 5 October 1955

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CLINICAL CHEMISTRY

Journal of the American Association of Clinical Chemists

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REAGENTS and REACTIONS 2

recent progress

For steroid hormone assay, enzymatic digestion of blood and urine often proves superior to old-fashioned acid hydrolysis. With β-glucuronidase the steroid nucleus can be split from its glucuronide conjugate: gently, specifically, accurately. At a recent Laurentian Hormone Conference, many investigators agreed on the advantages of this new method. But they also stressed the importance of using a reliable reagent. While bacterial and spleen glucuronidases may give inadequate, variable recoveries, participants from the University of Minnesota showed that "of all the glucuronidase preparations on the market," *Warner-Chilcott's Ketodase* (beef liver β-glucuronidase) was "the only fully effective preparation...."[1]

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a word of caution

The new radioactive tracers help in many research problems but not necessarily in the hospital laboratory, according to D. A. MacFadyen, (Rush Prof. of Biochemistry, U. of Ill. Coll. of Med.). Specifically, "measurement of blood volume by means of erythrocytes labeled with radioactive isotopes... offers no advantages over measurement by means of dyes, T-1824 for example."[2] *Warner-Chilcott's Evans Blue (T-1824)* matches any tracer technic in accuracy and has never shown toxicity in over 30 years of clinical experience. On the other hand, carcinogenic activity of P^{32} and protein changes in albumin made radioactive with I^{131} were recently reported.[3,4] The Evans Blue technic, "readily mastered by the average technician,"[5] is outlined in a new simplified procedure booklet (check coupon below).

1. Recent Progress in Hormone Research, New York, Academic Press, Inc., 1954, vol. 9, pp. 44, 411, 440. 2. Yearbook of Pathology and Clinical Pathology, Chicago, The Year Book Publishers, Inc., 1953-1954, p. 262. 3. J. Clin. Investigation *32*:246 (Aug.) 1953. Ibid. *33*:136 (Feb.) 1954. 4. Proc. Soc. Exper. Biol. & Med. *86*:266 (June) 1954. 5. Surg., Gynec. & Obst. *97*:709 (Dec.) 1953.

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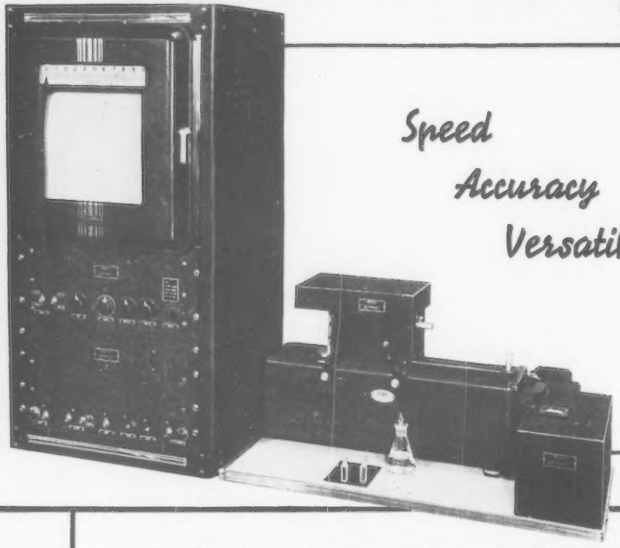

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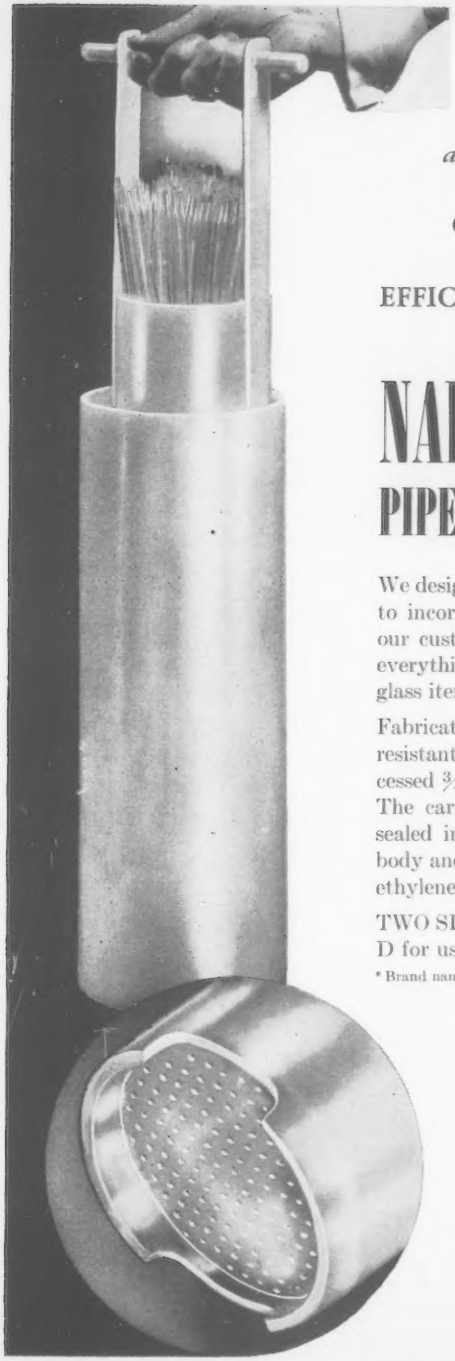

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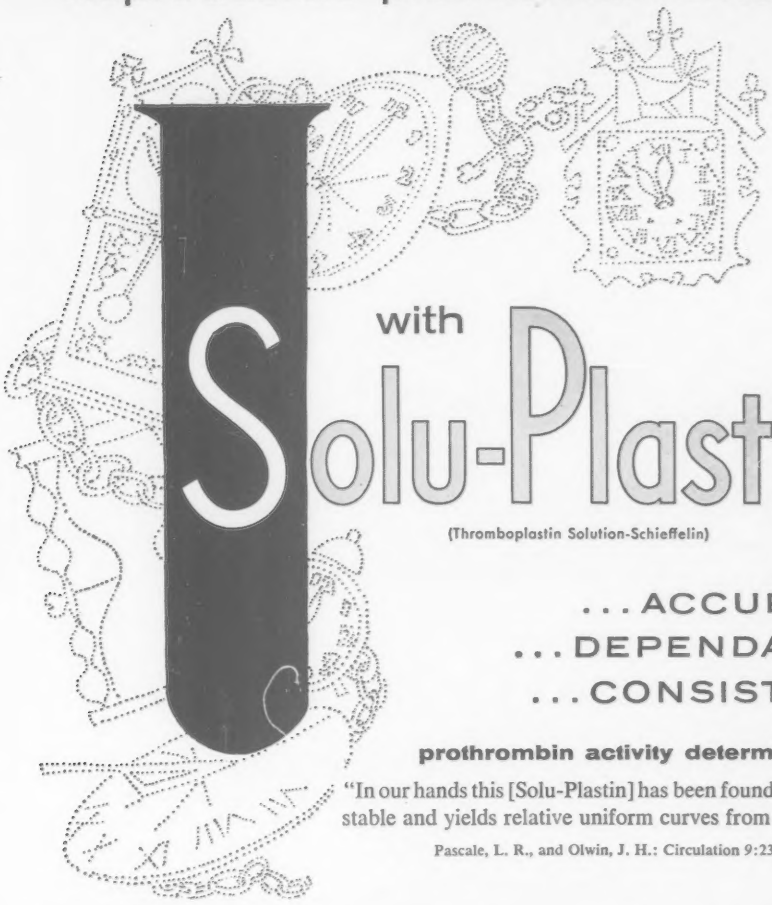

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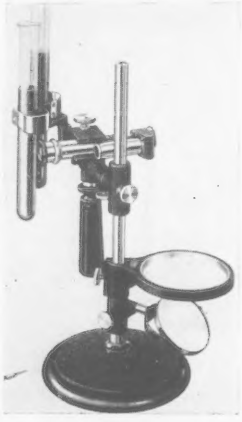

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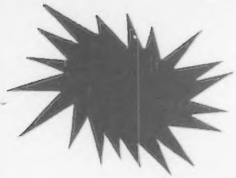

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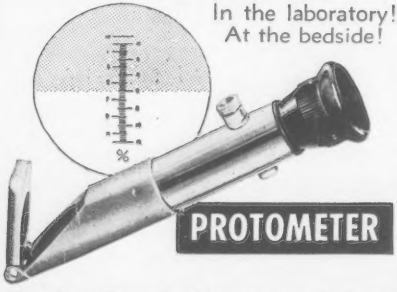

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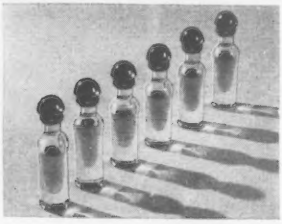

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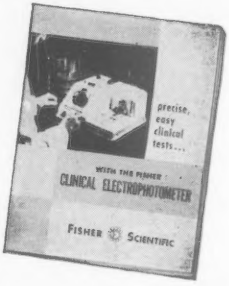

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Simultaneous Determination of pH, CO_2 Content, and Cell Volume in 0.1 ml. Aliquots of Cutaneous Blood

A Modification of the Shock and Hastings Technic

Richard B. Singer, Jane Shohl, and Dolores B. Bluemle

In 1934 SHOCK AND HASTINGS (1) described the construction and use of a micropipet, by means of which blood in 0.1 ml. aliquots could be used for the simultaneous determination of *p*H, CO_2 content, and red cell volume. In this method the blood in two pipets was diluted to 2.0 ml. with saline-phenol red solution, centrifuged, and used first for the determination of hematocrit value, second for the colorimetric determination of *p*H by visual comparison with previously prepared standards, and last, for the determination of CO_2 content in whole blood in the manometric Van Slyke apparatus. One or two additional pipets were filled with blood and 0.9% NaCl to serve as color blanks. A sample size of 0.5 ml. or less is thus sufficient for the determination of all three factors in duplicate, and cutaneous blood may therefore be utilized as an easily obtainable substitute for arterial blood. The method is particularly useful for the study of acid-base balance in infants.

Although the original method is suitable for most clinical purposes, the

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following modifications were found, after a thorough trial, to offer certain advantages in operation and improvements in accuracy. The need for the preparation and checking of a large set of bicolor standards has been eliminated by adapting the micropipet in such a way that the optical density of the unknown can be compared with the optical density of a phenol red solution in phosphate buffer of known *p*H, in a suitable filter photometer. A similar method of photometric estimation of *p*H in blood or plasma in standard colorimeter tubes has been described by Van Slyke, Weisiger, and Van Slyke (2). To obviate the difficulty of filling the stem of the micropipet with blood under oil, without accidental admixture of the latter, we utilized the method of collection and handling of blood proposed by Lilienthal and Riley (3). In our hands this resulted in a more accurate measurement of the 0.1 ml. aliquot of blood, a crucial factor in any microtechnic. Since the technic thus modified has proved its value in five years of use at the Hospital of the University of Pennsylvania, it has seemed worthwhile to describe the modifications in detail to encourage a wider clinical application of the technic. Evidence will also be presented to support the accuracy of the determinations and the validity of handling blood samples in a way that appears to violate traditional anaerobic methods.

RELATIONSHIP BETWEEN *p*H AND PHOTOMETRIC MEASUREMENT

The basic principles involved in the adaptation of a *p*H method utilizing indicators from a visual colorimetric to a photometric measurement have been reviewed by Van Slyke *et al.* (2). Phenol red behaves like a monovalent weak acid, so that the dissociated salt and undissociated acid components are related to the *p*H of the solution in the familiar Henderson-Hasselbalch equation:

$$pH = pK'_{PR} + \frac{(PR^-)}{(HPR)} \qquad (1)$$

The total concentration of indicator, (PR), or the sum of (PR^-) and (HPR), is kept very much less than the final buffer concentration of the mixture of the indicator solution with a test solution, such as blood or phosphate. It is therefore possible for the salt-acid ratio of the indicator to adjust to the *p*H of the test solution virtually without alteration of the latter.

Indicators like phenol red have the further property in both the dissociated and undissociated forms of possessing distinctive colors in solution. The alkaline form, PR^-, of phenol red is a deep purple color, and

the acid form, HPR, is yellow. Each form has a characteristic spectrophotometric absorption curve (4). In the *p*H range 6.7 to 8.6, mixture of the salt and acid forms gives a continuous series of intermediate colors, which can be analyzed visually by comparison with prepared standards or spectrophotometrically by making absorption curves or readings at two crucial wavelengths. The absorption curve for the alkaline phenol red has a maximum at $\lambda = 560$ mμ, and that for the acid form, a maximum at $\lambda = 430$ mμ, with virtually zero absorption at 560 mμ.

A reading of optical density or light extinction on a phenol red solution varies almost linearly with the concentration of alkaline phenol red, when a filter photometer with a green filter transmitting at about 540 to 560 mμ is used. The diameter of the colorimeter tubes and other parameters must be constant to maintain this relationship. In solutions in which the total phenol red concentration is known and constant, the photometer reading can therefore be related to *p*H through the concentration, (PR^-), and the factor pK'_{PR} in Equation 1. A series of phosphate buffer solutions containing the proper amount of phenol red is read in the photometer, and a calibration curve is prepared for optical density or extinction against the known values of *p*H (2). It is convenient to use a photometer such as the Klett-Summerson, in which the scale reading is proportional to optical density rather than to transmission.

APPARATUS

Micropipets of the Shock and Hastings design, with certain dimensions specified so that the pipets can be easily centrifuged and also placed in a uniform and suitable position in the photometer. Critical dimensions of the micropipets used originally in our work are shown in Fig. 1. This design required the drilling of a hole at the bottom of the cuvette or tube holder of the photometer, and insertion of a cork spacer on the inside of the bottom cover for the purpose of aligning the pipet bulb vertically with the light aperture of the holder (see Fig. 1). The hole does not interfere with the use of the ordinary photometer tubes in the holder. However, to avoid the need for this modification, the micropipet has been redesigned by the supplier. The model currently available fits directly into a metal adapter which can easily be inserted into or removed from the standard tube holder of the Klett-Summerson filter photometer.[1]

A filter photometer, capable of receiving the pipets and matching tubes of about 13 mm. outside diameter. A Klett-Summerson instrument was

[1] The adapter and micropipets made in accordance with such specifications are obtainable from Arthur H. Thomas Co., Philadelphia, Pa.

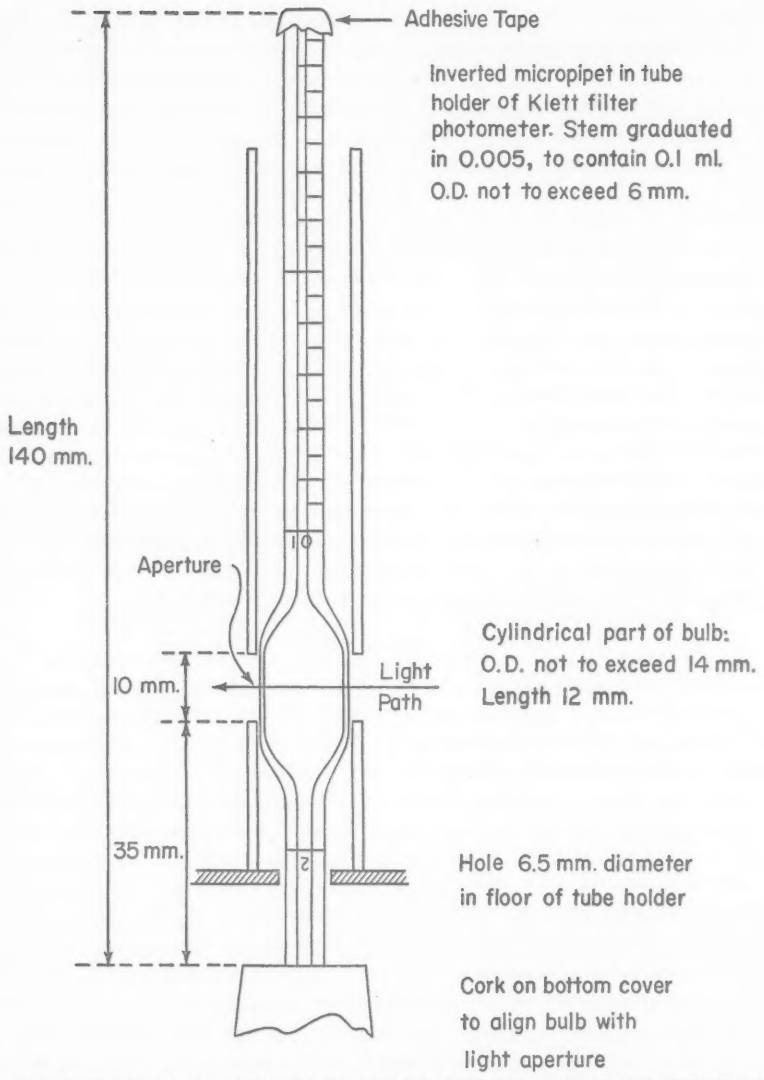
Adhesive Tape
Inverted micropipet in tube holder of Klett filter photometer. Stem graduated in 0.005, to contain 0.1 ml. O.D. not to exceed 6 mm.
Length 140 mm.
Aperture
10
Cylindrical part of bulb: O.D. not to exceed 14 mm. Length 12 mm.
Light Path
10 mm.
35 mm.
2
Hole 6.5 mm. diameter in floor of tube holder
Cork on bottom cover to align bulb with light aperture

Fig. 1. Sketch in cross-section, showing principal dimensions of original modification of the Shock and Hastings micropipet, and position of micropipet in the cuvette holder of the Klett-Summerson filter photometer. The walls of the cylindrical part of the bulb should be of clear glass, of uniform thickness, and free of defects. The actual outside diameter

used in all of our work.[2] Although the micropipet was modified specifically for use in this photometer, it might be used in any filter photometer or spectrophotometer, provided that it could be positioned with the light path restricted to the cylindrical part of the bulb.

Test tubes for use as photometer tubes, matched as nearly as possible to the internal diameter of the micropipets (see specifications in Fig. 1). These can be selected from a quantity of round-bottom Pyrex test tubes of outside diameter 12.5 mm. and 100 mm. length.

A small water bath set at 38° and provided with a rack to hold micropipets and small photometer tubes.

A centrifuge with four brass shields (cups) of standard outside dimensions about 120 mm. by 19 mm. An angle centrifuge cannot be used because the stem of the micropipet is likely to break. A satisfactory small centrifuge is the Clay-Adams Standard Safeguard centrifuge, Model No. CT-1110, having a maximum speed of 2600 rpm.

Heavy rubber bands, approximately 75 mm. long and 6 mm. wide.

Syringes. A 1-ml. tuberculin syringe and 26-gage hypodermic needle for measuring the heparin solution; several 2-ml. syringes for collecting blood samples, with metal caps (Becton-Dickinson No. 425A); several 20-ml. syringes for storing solutions.

Collecting tips for the 2-ml. syringes, made from a piece of single bore #00 rubber stopper about 8 mm. in length, with a hollow cup ground in one end.

Adapters for use in filling the micropipet from the 2-ml. or 20-ml. syringes, made from pressure tubing of ⅛″ bore and ⅛″ wall. The pieces are 10 to 12 mm. in length and must be cut with a sharp knife so that the ends are flat.

Rubber adapters to center the stem of the micropipet in the centrifuge

[2] The instrument available for our use happened to be an early model that differs from current models in its optical system and in the galvanometer. For ease in reading, a box-type spot galvanometer had been substituted for the needle galvanometer that is a standard part of the Klett-Summerson instrument. However, the pipets have been successfully used in many other unmodified photometers of this manufacture. Any model can be used as long as the light aperture of the adapter, when in position, is within the light aperture of the instrument.

(O.D.) of the bulb averaged about 13 mm. A further improvement in design, made by Arthur H. Thomas Co., is incorporated in the current model of the micropipet. The neck has been shortened so that it is unnecessary to modify the Klett instrument by drilling a hole in the floor of the tube holder, as shown above. A removable metal sleeve has also been made available to slip into the tube holder. The purpose of this is to ensure accurate dimensions of the aperture and proper centering of the pipet.

shield. For use in the shields specified previously, these are made from pressure tubing of ¼″ bore and 3/16″ wall, and are 15 mm. in length.

CALIBRATION

The volume contained in the stem of each micropipet to the nominal 0.1 ml. mark should be checked in order to insure accuracy of sample measurement. This calibration can be carried out to the nearest 0.0005 ml. by weighing the measured volume of mercury, by titrating with dilute alkali the measured volume of standard 1N acid and several rinsings of the stem, or by use of a calibrating microburet.

The micropipets and selected matching test tubes must also be calibrated with respect to their photometer reading, at both low and high readings. The micropipet is placed in the holder bulb down, and pipets and test tubes are always rotated so that the identifying mark faces the front of the instrument. With the green filter in place (Klett No. 54) readings are made (a) with water and (b) with an alkaline phenol red solution of 3.75 mg./l. or one of similar optical density. It is convenient in estimating the water corrections to make the original balance point with a scale setting of +10 divisions on the Klett instrument, since readings are not possible on the negative side of the zero setting. *Failure to reproduce scale readings within one or two divisions on the Klett, or large correction values, may indicate that the aperture is not centered properly with respect to the cylindrical part of the bulb.*

REAGENTS

1. For Collection of Blood

Mercury, clean and free of acid or alkali.
Heparin sodium, 1% in 0.9% NaCl solution (Abbott No. 3672).

2. For Hemoglobin Determination

0.1% Na_2CO_3 solution (1 Gm. per liter).
0.012M $CuSO_4 \cdot 4NH_3 \cdot H_2O$. Dissolve 2.998 Gm. of $CuSO_4 \cdot 5H_2O$ and dilute to 1 liter with approximately 2M NH_4OH.

3. For Use in Micropipets

Phenol red, 0.1% stock solution. Grind 1 Gm. of phenol red with 28.2 ml. of 0.1N NaOH until dissolved and dilute to 1 liter with distilled water. Filter if necessary. Allow to stand several days before using. Store in refrigerator.

Phenol red, 0.0075% working solution. Dilute 75 ml. of 0.1% phenol red to 1 liter with boiled distilled water. Store in refrigerator.

NaCl, 18% stock solution. Dissolve 180 Gm. of NaCl and dilute to 1 liter with distilled water. Store in refrigerator.

Saline-phenol red solution. Dilute 10.5 ml. of 0.0075% phenol red and 5.0 ml. of 18% NaCl to a total of 100 ml. with boiled distilled water. This solution should be freshly prepared at least once a week and, until used, is stored in a closed container or in a 100 ml. buret with a soda lime tube in the top.

4. For CO_2 Determination

(as described in Peters and Van Slyke (5))

Caprylic alcohol.

1N Lactic acid. CO_2-free stock solution, stored in refrigerator.

0.1N Lactic acid. Prepare fresh, free of CO_2 and air.

18N Sodium hydroxide (saturated). CO_2-free stock solution.

1N or 5N Sodium hydroxide. Prepare CO_2-free by dilution of 18N sodium hydroxide with boiled distilled water. The 1N solution must be fresh and air-free. The 5N solution is more stable because of lower solubility for gases (5, p. 284), but causes more rapid stopcock deterioration.

BUFFER STANDARDS AND CALIBRATION CURVE FOR pH MEASUREMENT

Stock solutions of M/3 Na_2HPO_4 (47.33 Gm./L.) and M/3 KH_2PO_4 (45.36 Gm. per liter). These solutions are prepared by dissolving the anhydrous reagent grade salt[3] in freshly boiled redistilled water, and are stored in the refrigerator in glass-stoppered Pyrex bottles. This concentration has been reduced from that used by Van Slyke *et al.* (2)[4] to lessen the amount of crystalline precipitation in the cold. Since the alkaline Na_2HPO_4 tends to pick up CO_2 if exposed to room air, the bottles are unstoppered only at the time of preparing desired solutions. The stock solutions appear to be stable for at least two years.

M/3 Mixed phosphate solutions. These are prepared in the proportions indicated in Table 1, so that the indicated *p*H will be obtained when each solution is diluted 1 to 5 to a final phosphate concentration of M/15. At 20° the *p*H will be 0.03 higher than the figures shown in Table 1 for 37°.

[3] The Merck Company provides anhydrous salts of high purity specially processed for buffer solutions.

[4] The concentrations in Gm./L. given by Van Slyke *et al.* on page 746 of this reference (2) actually refer to molar concentrations, not 0.5 molar, as stated.

Table 1. STANDARD M/15 PHOSPHATE BUFFERS
Based on data of Hastings and Sendroy (6)

*p*H[a] at 38°C.	pK_2'[a] at 38°C.	*R*[b]	Na_2HPO_4[c](mM/L.)	KH_2PO_4[c](mM/L.)	μ[d] (ionic strength)
6.79	6.79	1.000	33.3	33.3	0.133
6.80	6.79	1.025	33.7	32.9	0.135
7.00	6.78	1.660	41.7	25.0	0.150
7.20	6.77	2.68	48.6	16.1	0.164
7.40	6.76	4.35	54.3	12.4	0.175
7.60	6.75	7.05	58.3	8.3	0.183
7.80	6.74	11.42	61.3	5.4	0.189
8.00	6.73	18.50	63.3	3.4	0.193

[a] Electrometric measurements with the H_2 electrode, referred to a *p*H of 1.08 for 0.1*N* HCl at 38°. The same values can be used at 37°.

[b] *R* is the molar ratio of Na_2HPO_4 to KH_2PO_4 used.

[c] These figures for final concentration of the buffer components, or any suitable multiple or fraction thereof, can be used as the number of ml. of each M/15 salt solution required for the preparation of the M/15 phosphate buffer of stated *p*H.

[d] The value of pK_2' is affected by variations in the ionic strength, μ, defined as

$$\tfrac{1}{2} \Sigma\, c_n V_n^2,$$

where c_n is the concentration of each ion, in Eq./L., and V_n is the valence of that ion.

The column headed *R* in Table 1 gives the molar ratio of Na_2HPO_4 to KH_2PO_4. If a mixed phosphate solution to make a *p*H 7.40 standard is desired, 10.00 ml. of M/3 KH_2PO_4 solution should be mixed with 10.00 *R* or 43.5 ml. of M/3 Na_2HPO_4. If a final volume of 66.7 ml. is sufficient for each mixed phosphate solution, the figures for the ultimate concentrations of Na_2HPO_4 and KH_2PO_4 (in mM/L., Table 1, columns 4 and 5) can be used for the number of milliliters for each solution. These solutions are also stable if stored in Pyrex bottles in the refrigerator. The *p*H and pK_2' values corresponding to a given Na_2HPO_4/KH_2PO_4 ratio in Table 1 are taken from the experimental results of Hastings and Sendroy (6) and are based on H_2 electrode measurements at 38°. They are identical with the ones used by Van Slyke *et al.* (2). There is no significant difference in pK_2' due to use of a temperature of 37° rather than 38°.

Standard M/15 phosphate buffers. Measure 20.00 ml. of mixed phosphate buffer, 10.00 ml. of the working solution of phenol red (75 mg./L.) into a 100 ml. volumetric flask and dilute to 100.0 ml. with freshly boiled redistilled water. This gives a standard M/15 phosphate of the designated *p*H at 37° containing 7.5 mg./L. of phenol red. Although such standard solutions are not so stable as the more concentrated phosphate mixtures, we have observed no significant change in photometer reading and *p*H over periods as long as two months, if the solutions are kept in Pyrex bottles in the refrigerator when not in use.

Calibration curve. At least two calibrated pipets or test tubes are filled for each standard buffer solution. If the pipets are used, the tips are dried and covered with adhesive tape as described in "Procedure." It is convenient to use one of the matched test tubes as a permanent water blank for the zero setting on the photometer in lieu of a calibrated pipet. Each buffer solution must be heated to 38° before reading in the photometer with a green filter, maximum transmission 540 mμ. In the length of time necessary to place a pipet or test tube in the photometer and obtain a reading, the fall in temperature is generally no more than 1°. The zero setting is checked with the water blank after each reading; readings may be repeated, if desired, after rewarming. The appropriate pipet or tube correction is subtracted from each reading, and a graph is constructed of the mean photometer reading against *p*H.

It has been our practice to recheck the complete calibration curve only at infrequent intervals. However, the level of the curve is checked with each *p*H determination by reading at least one buffer, usually the one at *p*H 7.40, and a stable, colored reference solution, the $CuSO_4 \cdot 4NH_3 \cdot H_2O$ used in the hemoglobin determination. The level of the curve set by this buffer reading usually does not vary more than two or three scale divisions from day to day.

Because the pK′ of phenol red in the saline-plasma mixture differs slightly from the pK′ in phosphate buffer, it becomes necessary to apply a correction to the *p*H reading of the unknown solution to make it correspond to the buffer calibration curve. The pK′ of phenol red in various salt solutions has been accurately determined by Sendroy and Hastings (7); values for the M/15 phosphate buffers and 0.9% NaCl solution (with

Table 2. pK′ OF PHENOL RED—SALT CORRECTIONS
Based on data of Sendroy and Hastings (7)

*p*H at 38°C.	*p*K′ PO_4 buffer	*p*K′ 0.9% NaCl	Difference[a]	*p*H Range at 37°C.	Correction[b]
6.80	7.658	7.618	+0.040	under 6.94	−0.04
7.00	7.659	7.628	+0.031	6.94–7.18	−0.03
7.20	7.660	7.637	+0.023	7.19–7.42	−0.02
7.40	7.660	7.645	+0.015	7.43–7.67	−0.01
7.60	7.662	7.651	+0.011	7.68–7.91	0.00
7.80	7.660	7.657	+0.003	over 7.91	+0.01
8.00	7.653	7.662	−0.009		

[a] Difference between pK′ of phenol red in M/15 phosphate buffer and pK′ in a solution containing 124 mM/L., NaCl and 30 mM/L. $NaHCO_3$, at the stated *p*H.

[b] Correction to be applied to the *p*H read with 0.9% NaCl solution as a diluent, in order to allow for the difference in pK′ of phenol red in this solution as compared with the M/15 phosphate buffer standards.

H_2CO_3-$NaHCO_3$ buffer) are shown in Table 2, as taken from their work. The correction is given for various ranges of observed *p*H. Ordinarily this correction has been ignored as negligible in the past, but for the most accurate work it should be applied.

PROCEDURE

A syringe of 2 ml. capacity, graduated in 0.1 ml., is used for the blood collection. A constant amount, 0.05 ml., of heparin saline solution is measured in a tuberculin syringe and then injected through a 26-gage needle into the barrel of the 2 ml. syringe. After ejection of the air, about 0.3 ml. of mercury is drawn into the syringe and the rubber collecting tip is attached to it.

Two greased syringes of 20 ml. capacity are used for filling the micropipets with diluent. A satisfactory grease for the syringes is Arthur H. Thomas stopcock grease; silicone grease is too alkaline for this purpose. About 2 or 3 ml. of mercury are drawn into each syringe. Saline, 0.9%, to be used in the control pipets, is drawn into one syringe and the saline-phenol red solution into the other. A rubber adapter which will serve as a connecting piece between syringe and pipet is attached to each syringe. Before filling the pipets, the saline-phenol red solution is adjusted visually to *p*H 7.4 $\pm$ 0.2 by adding a fractional drop of dilute alkali or acid.

Collection of Blood Sample

To produce peripheral vasodilatation and fully "arterialize" cutaneous blood, the patient's hand (or the heel in infants) is first placed in warm water at 45° for 3–5 minutes. A sterilized spring lancet or blade is used to make the finger cut. Blood is collected in the rubber funnel tip as it falls drop by drop from the finger and is drawn into the syringe. The flow of blood should be free, but gentle pressure may be applied if necessary. The initial air bubble, if any, is ejected and the syringe is shaken gently to mix the blood with the heparin as the collection proceeds. About 1 ml. of blood should be collected in 1–3 minutes. If the first cut does not supply enough blood for the sample, a second cut may be made and the blood pooled with that of the first cut. The total amount of blood must be noted to the nearest 0.05 ml. so that the heparin dilution can be calculated. The rubber collecting tip on the syringe is replaced by a metal cap. The syringe is placed cap down in a beaker of ice. When the procedure is properly carried out, the exposure of blood to air during collection is without significant effect on the *p*H and CO_2 content.

Instead of the fingertip, the ear lobe can be used as a source of cutane-

ous blood, after warming by dry heat as described by Lilienthal and Riley (3).

Determination of Hemoglobin Concentration[5]

The collecting syringe is shaken gently for thorough mixing. The metal cap is removed and the blood in the syringe tip is discarded. A rubber syringe adapter is attached to the syringe tip. A drop of blood is forced from the syringe and is drawn by suction into a 0.02 ml. (20 cu.mm.) pipet calibrated to contain that amount. This sample is delivered into a photometer tube containing 5.0 ml. of 0.1% Na_2CO_3 solution. The pipet is rinsed several times with the Na_2CO_3 solution and the tube is stoppered and inverted repeatedly. A tube containing 0.012M $CuSO_4 \cdot 4NH_3 \cdot H_2O$ is used as a standard. The solutions are read in a photometer against a distilled water blank. A 540 mμ (green) filter is used.

$$\text{Hb, in mM/L.} = \frac{\text{Reading Hb}}{\text{Reading } CuSO_4 \cdot 4NH_3 \cdot H_2O} \times \frac{\text{volume blood} + \text{volume heparin}}{\text{volume blood}} \times 9.34$$

$$\text{Hb, in Gm./100 ml.} = \frac{\text{Reading Hb}}{\text{Reading } CuSO_4 \cdot 4NH_3 \cdot H_2O} \times \frac{\text{volume blood} + \text{vol. heparin}}{\text{volume blood}} \times 15.6$$

The factors 9.34 and 15.6 in these equations are derived from the data of Drabkin (8), who proposed the use of $CuSO_4 \cdot 4NH_3 \cdot H_2O$ as a standard solution for photometric estimation of hemoglobin and other pigments. In the Klett-Summerson photometer with a 540 mμ filter, Drakbin found a ratio of 0.963 for the reading of a standard hemoglobin solution (8.98

[5] A detailed description is given for the hemoglobin determination because this is a slightly better index of blood nonbicarbonate buffers than is the packed cell volume, and because the reference standard can be used to check the photometer reading of the phenol red solutions. In our hands the copper sulfate photometric standard was stable and reproducible, and gave good results. However, more recent work by Drabkin and others on behalf of the National Research Council has indicated that slight variations may occur in the water content of the crystalline $CuSO_4 \cdot 5H_2O$ that make it undesirable as a primary photometric standard. Published recommendations have appeared in this journal, Vol. 1, page 151, to determine hemoglobin as cyanmethemoglobin, with the use of a stable, primary cyanmethemoglobin standard that is now available through Division of Medical Sciences, National Academy of Sciences–National Research Council. The use of this new standard is to be preferred.

mM./L. or 15.00 Gm./100 ml., diluted 1–251) and the reading of 0.012M copper sulfate standard. The reading of the standard, which varies somewhat from instrument to instrument, and serially in the same instrument, therefore corresponds to that of a solution containing 8.98/0.963 = 9.34 mM./L. of hemoglobin, or 15.00/0.963 = 15.6 Gm./100 ml. of hemoglobin, after dilution 1–251.

Filling of Micropipets

Before filling each micropipet with an aliquot of the blood sample collected, the collecting syringe is shaken gently and a small portion of blood is discarded by touching a clean cloth to the blood surface. The calibrated end of the micropipet is placed end to end against (not inside) the rubber adapter, and blood is forced carefully from the syringe until the pipet is filled slightly beyond the 0.1 ml. mark. Manipulation is facilitated by holding the barrel of the syringe and the pipet in the left hand while the right hand slowly pushes the syringe plunger (Fig. 2). When the pipet has been filled, it is grasped by the right hand with the forefinger covering the open stem above the bulb; contact with the syringe is then broken. The blood is adjusted to the 0.1 ml. mark by drawing off the excess with a clean cloth or by gently tapping the pipet end with a finger. The pipet is

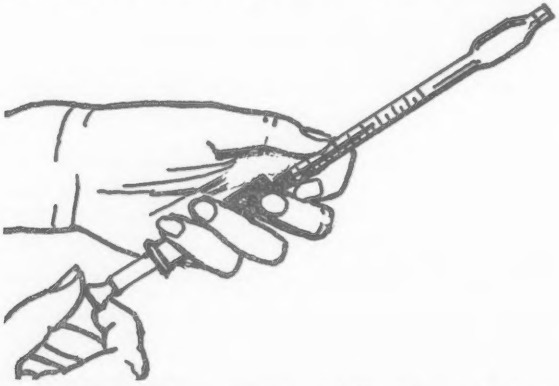

Fig. 2. Method of filling micropipet with blood from a 2-ml. syringe. The stem of the pipet is held between the thumb and forefinger of the left hand. The barrel of the syringe is held between the palm and fourth and fifth fingers of the same hand. The flat bottom of the pipet stem must be firmly apposed to the flat end of the rubber adapter on the tip of the syringe. Filling is controlled by gentle pressure on the plunger of the syringe with the right hand. The same procedure is used in filling the micropipet to the 2.0 ml. mark with diluent from a 20-ml. syringe.

then tilted slightly, bulb down, until there is a small air space between the blood and the calibrated end of the pipet. The outside of the calibrated end is wiped clean.

Saline-phenol red solution in the greased 20 ml. syringe is used as diluent for three pipets, and 0.9% NaCl for the fourth, which serves as a color blank for the *p*H determination. The dilution is accomplished by the filling technic just described; but to avoid trapping air in the pipet bulb, the syringe and pipet should be gradually raised to a vertical position while filling and then lowered to a horizontal position when the solution is slight above the 2.0 ml. mark. Care must be taken to hold the pipet firmly against the rubber adapter, maintaining the pressure fit. After adjusting the volume, a small air space is again left in the end of the pipet. The outside is dried carefully, and the tip of the calibrated end is sealed with a small piece of adhesive tape.

Centrifuging and Determination of Packed Red Cell Volume (Vc)

One of the heavy rubber bands is placed lengthwise about each pipet and flat over the adhesive tape seal to protect the pipet in centrifuging. The calibrated end of the pipet is placed in the cup adapter made from rubber pressure tubing. The pipets in this way are held firmly in the centrifuge cups. Centrifugation is carried out at 2600 rpm for 15–20 minutes. The rubber bands should be removed carefully after centrifugation since they sometimes stick to the adhesive tape seal.

The volume of packed red cells (*Vc*), noted to the nearest 0.5 per cent in the calibrated stem of the pipet, must be corrected for the heparin dilution.

$$\text{Vc} \times \frac{\text{volume blood} + \text{volume heparin}}{\text{volume blood}} = \text{corrected Vc}$$

pH Determination

To check against the standard curve, two pipets or test tubes are filled with *p*H 7.40 phosphate buffer containing phenol red. All the pipets and tubes except the saline blank are heated in a water bath to 38°. Readings are taken on each pipet after placing it bulb down in the holder of the photometer, checking stem orientation each time. A green filter with maximum transmission at 540 mμ is used. All readings should be taken promptly or the red cells in the stem of the inverted pipet may flow into the bulb.

The reading of each micropipet must be corrected by applying the

appropriate pipet calibration. The final corrected reading, R, is calculated as $R = R_2 - R_1$, where R_2 is the corrected reading for the blood-phenol red sample and R_1 is the corrected reading for the blood-saline blank. The pH is estimated by the interpolation of R on the standard curve of the pH of the phosphate buffers at 37° versus the photometer scale reading. This value, plus the appropriate pK′ correction (see Table 2), gives the final pH value.

Determination of Whole Blood CO_2 Content $(CO_2)_b$

The CO_2 content of the blood diluted with saline-phenol red is determined in a Van Slyke manometric blood gas apparatus by the basic method of Van Slyke and Neill, as adapted to the characteristics of the sample (1, 5).

One drop of caprylic alcohol is drawn into the capillary and 3 ml. of 0.1N lactic acid are measured in the cup of the apparatus. After removing the adhesive tape seal from the pipet, a small piece of rubber tubing is placed on the tip of the calibrated end to provide a watertight seal between the pipet and the lactic acid. The contents of the pipet are delivered directly into the extraction chamber, delivery being halted with a small air bubble in the stopcock capillary. The air bubble is dislodged with a wire dipped in caprylic alcohol, and 2.5 ml. of the lactic acid are drawn into the machine. The stopcock is sealed with mercury and the mercury level in the chamber is lowered to the 50 ml. mark. After extraction the CO_2 is reabsorbed with 1 ml. of 1N NaOH. Pressure readings are taken at the 0.5 ml. volume.

A blank CO_2 determination is made in the same manner on a 2.0 ml. sample of the saline-phenol red solution used to dilute the blood.

The difference in pressure due to the blood, ΔP, is calculated by subtracting the blank correction from the difference in the pressure readings for the blood diluted with saline-phenol red, $P_1 - P_2$ (see "Sample calculations").

The CO_2 content of the blood in mM/L., $(CO_2)_b$, is obtained by multiplying the pressure difference, ΔP, by the appropriate factors for temperature, pipet calibration, and heparin dilution.

$$(CO_2)_b = P \times \frac{\text{Temperature Factor}}{\text{Pipet Factor}} \times \frac{\text{volume blood} + \text{volume heparin}}{\text{volume blood}}$$

Temperature factors as calculated by Peters and Van Slyke (5) are reproduced in Table 3, page 304.

Centrifugation of Excess Blood for Determination of Plasma Constituents

Additional determinations, such as plasma Na, K, Cl, and CO_2 can be made if a larger sample of blood (1.5 to 2.0 ml.) is collected at the time of the finger or ear lobe puncture. When it is planned to collect a blood sample larger than 1.2 ml., it is advisable to grease the plunger of the collecting syringe before adding the heparin saline solution.

After the aliquots required for the *p*H, CO_2, and hemoglobin determinations have been taken from the syringe, the contents are mixed; the syringe is held vertically, tip down, while the mercury is carefully ejected. The blood is transferred to the bottom of a 1-ml. micro centrifuge tube through a 2-inch 21-gage needle. The centrifuge tube should be filled from the bottom to avoid trapping an air bubble. A small rubber stopper pierced by a short 24-gage hypodermic needle is quickly inserted in such a way that any trapped air and a small amount of excess blood are forced up through the needle. The needle is then removed and the tube is ready for anaerobic centrifugation. The brief exposure of the blood to room air while the tube is being filled does not result in a significant change in *p*H or CO_2 content, as is shown later. After centrifugation the stopper is removed and the plasma is carefully withdrawn through a 21-gage needle into a greased 2-ml. syringe. The empty needle is removed, the syringe held tip up, and the air bubble (from the needle) is ejected. The plasma can be stored anaerobically if the syringe tip is protected with a metal cap.

Sampling is accomplished by forcing a drop of plasma up into a collecting piece placed on the syringe tip and drawing up a 0.05 or 0.1 ml. aliquot into a suitable type of calibrated pipet such as the Linderstrom-Lang-Hølter semiautomatic constriction pipet. The pipet designed by Kirk is also suitable and in the sizes from 0.05 to 0.2 ml. can be utilized as an accurate delivery pipet despite the fact that it is designed "to contain."[6] In this pipet the volume to deliver is about 2 per cent less than the contained volume. Filling and delivery in these pipets are accomplished with the aid of mouth suction and pressure through a rubber tube and mouthpiece, or with the aid of a pipet controller (9).

If desired, the plasma CO_2 content can be measured, with 0.1 ml. aliquots delivered under water in the cup of the Van Slyke apparatus as described previously. The relative accuracy of this determination is greater than that of the whole blood CO_2 because the blank correction is

[6] The Linderstrom-Lang-Hølter and Kirk micropipets used by us were obtained from Arthur H. Thomas Co., Philadelphia, Pa.

more stable and the concentration of total CO_2 is about 20 per cent higher in plasma.

Unless plasma CO_2 is to be included, all plasma samples can be drawn directly from the centrifuge tube. If a potassium determination is desired, the blood must be transferred to the micro centrifuge tube before icing the collecting syringe to prevent the exchange of potassium across cell membranes.

Plasma sodium and potassium concentrations can be measured with the internal standard flame photometer (10). For the determination of chloride we have used Sendroy's titrimetric iodate method, with modifications as described by Van Slyke and Hiller (11) on 0.05 ml. aliquots. Protein concentration could be estimated in one drop by means of the gradient tube (12) or falling-drop method (13). A variety of substances in plasma can be determined by suitable micro methods, up to the limit of plasma available, which would be about 0.5 ml. for a 1.5 ml. sample of blood.

Because the plasma is diluted with a heparin solution containing sodium and chloride, appropriate corrections must be applied to the measured concentrations of these ions, and to the concentrations of other substances estimated in heparinized diluted plasma. The corrections are calculated from the amount of heparin solution used in relation to the amount of plasma, and, in the case of sodium and chloride, from the concentration of these ions in the heparin solution.

SAMPLE CALCULATIONS

1. Dilution Factor

Suppose the total volume of blood and heparin diluent (measured in collecting syringe) to be 1.50 ml. Volume of heparin solution measured from tuberculin syringe—0.05 ml. Volume of blood = 1.50 − 0.05 = 1.45 ml. Consequently, the dilution factor for use in calculating hematocrit value and whole blood concentrations is 1.50/1.45 or 1.034.

2. Hemoglobin Concentration

Suppose that the corrected mean reading for the standard $CuSO_4 \cdot 4NH_3 \cdot H_2O$ solution in the Klett-Summerson photometer is 200, and the mean reading for the hemoglobin diluted 1/251 is 185. The hemoglobin pipets used have a calibrated volume of 20.2 cu.mm.

$$\text{Hb conc} = (9.34)\ (186/200)\ (20.0/20.2)\ (1.034) = 8.9 \text{ mM/L.}$$

3. Hematocrit Value

Suppose that the mean value in four micropipets is 0.440.

$$\text{Corrected Vc} = (0.440)\ (1.034) = 0.455$$

It is not useful to measure or calculate the hematocrit value more accurately than to the nearest 0.005.

4. Photometric pH

Suppose the mean reading of the plasma-indicator-saline mixture in three micropipets (with the individual pipet calibration applied to each reading) to be 156. The corrected blank reading is 10. The mean corrected reading for the *p*H 7.40 phosphate buffer is 152, and for the *p*H 7.20 buffer, 111.

Sample reading, corrected for blank = 156 − 10 = 146. The *p*H can then be read from a suitable graph on which photometer readings are plotted against *p*H, or estimated by the following equation in the *p*H range in which the relation is virtually linear:

$$p\text{H} = 7.40 - [(152\text{-}146)/(152\text{-}111)][0.20] = 7.40 - (6/41)(0.20) = 7.37$$

Finally, the correction from Table 2 must be applied (the difference of pK′ of phenol red in 0.9% NaCl solution as compared with M/15 phosphate buffer):

$$\text{Corrected } p\text{H} = 7.37 - 0.02 = 7.35$$

5. Whole Blood CO_2 Concentration

Assume the following data. Volume of micropipet = 0.099; calibration of chamber volume = 0.495 ml. Blank pressure difference for 2.0 ml. of saline-phenol red = 14.0 mm. Calculated blank for 1.9 ml. of saline-phenol red = (14.0)(1.9/2.0) or 13.3 mm. At 24° the two pressure readings obtained for the diluted blood sample are P_1 = 182.6 mm. and P_2 = 114.0 mm.

$$\text{Corrected } \Delta P = (182.6 - 114.0) - 13.3 = 68.6 - 13.3 = 55.3$$

The factor in Table 3 at 24°, for a chamber volume of 0.5 ml. and a sample of 0.100 ml. is 0.304.

$$(CO_2)_b = (55.3)(0.304)(0.100 \times 0.100/0.099)(0.495/0.500)(1.034)$$
$$= (16.8)(1.034) = 17.4 \text{ mM/L.}$$

It is convenient to prepare a table of temperature factors that includes

Table 3. Factors for Calculation of Whole Blood CO_2 Content

Temperature (°C.)	*Factor*[a] (mM/L./mm.)	*Temperature* (°C.)	*Factor*[a] (mM/L./mm.)
15	0.320	25	0.302
16	0.318	26	0.300
17	0.316	27	0.299
18	0.314	28	0.297
19	0.312	29	0.296
20	0.311	30	0.294
21	0.309	31	0.292
22	0.307	32	0.290
23	0.305	33	0.289
24	0.304	34	0.287

[a] Factor by which the gasometric pressure difference on the Van Slyke-Neill apparatus, in mm. of Hg, is to be multiplied to obtain the whole blood CO_2 content in mM/L. These factors are calculated for a sample size of 0.1 ml., a solution volume of 4.5 ml., a chamber volume of 0.5 ml., and an "i" factor of 1.037. See Peters and Van Slyke (5). These factors are more convenient to use than the ones given by Shock and Hastings (1), because they give directly the CO_2 concentration in the blood sample instead of the blood-saline mixture.

the correction for the chamber volume. The sample and pipet corrections must be calculated in each individual determination; the dilution factor is usually applied only to the mean of the replicate CO_2 determinations.

6. Obtaining the Derived Acid-Base Factors

The calculated CO_2 pressure and total CO_2 or bicarbonate concentration of plasma can be obtained from the nomograms of Van Slyke and Sendroy (14) and Hastings and Shock (15). The whole blood buffer base concentration can be obtained from the nomogram of Singer and Hastings (16), which also gives values for CO_2 pressure and plasma total CO_2 or bicarbonate. In the application of all of these nomograms to whole blood CO_2 concentrations it is necessary to use the hemoglobin concentration or hematocrit value as one auxiliary parameter, and a known or assumed O_2 saturation as the other. Unless the patient is cyanotic or the sample shows obvious signs of unsaturation, it is assumed that cutaneous or arterial blood is fully oxygenated. If the oxygen saturation is less than 90 per cent, slight errors in CO_2 pressure and buffer base concentration will be introduced unless account is taken of this factor. In the example cited here, with Hb 8.9 mM/L. or Vc 0.455 and an assumed 100 per cent saturation, the whole blood CO_2, 17.4 mM/L., is found in Scale 1 of the nomogram of Singer and Hastings, and the *p*H, 7.35, on Scale 4. A straight line is drawn through these two points and the derived variables are read off.

Plasma $(CO_2)_p$ = 21.0 mM/L. (right-hand edge, Scale 1)

Plasma $(HCO_3^-)_p$ = 19.8 mM/L. (Scale 2)

Whole blood $(BB^+)_b$ = 43 mEq./L. (Scale 3)

P_{CO_2} = 37 mm. (Scale 5)

This represents for an adult a mild metabolic acidosis with a CO_2 pressure at the lower edge of the normal range. However, in an infant, these acid-base conditions could all be within a normal range.

RESULTS

Accuracy of Modified Microtechnic

The respective errors of the various methods involved in the microtechnic have been estimated by an analysis of the deviations in a large number of sets of replicate determinations. The results in Fig. 3 show the distribution of the deviations from the mean. The standard error for the mean of duplicate hemoglobin determinations is ±0.029 mM/L. or less than half of one per cent of the average concentration, a relative error equal to that of the calibration of the 20 cu.mm. pipets. The standard error of the mean of quadruplicate packed cell volumes was ±0.0023. The standard error of the *p*H in triplicate was ±0.0051, and that of the whole blood CO_2 content in duplicate ±0.16 mM/L. Confidence limits have been calculated on the basis of the difference between means (obtained with the usual number of replicates) that has only a 5 per cent probability of being due to chance variation; the ratio of this difference to the standard error of the difference, or $\sqrt{2} \times$ standard error, is 1.96 (17). This least significant difference is less than 0.1 mM/L. or 0.15 Gm./100 ml. for hemoglobin, 0.006 for cell volume, 0.014 for *p*H and 0.44 mM/L. for whole blood CO_2 content. The accuracy of this whole blood CO_2 determination can be compared with that of the determination of CO_2 content in 0.2 ml. aliquots of plasma, measured in a stopcock pipet. The standard error of the duplicate plasma determination was ±0.11 mM/L., and the least significant difference 0.3 mM/L.

Effect of Collection or Transfer of Blood with Momentary Exposure to Air

In Table 4 results are presented of a representative experiment in which heparinized blood was transferred dropwise from a 20-ml. syringe to four separate 2-ml. syringes in such a way as to simulate the collection of cutaneous blood. Two of the small syringes (A and C) were previously prepared with 0.05 ml. of heparin saline solution, and two (B and D) con-

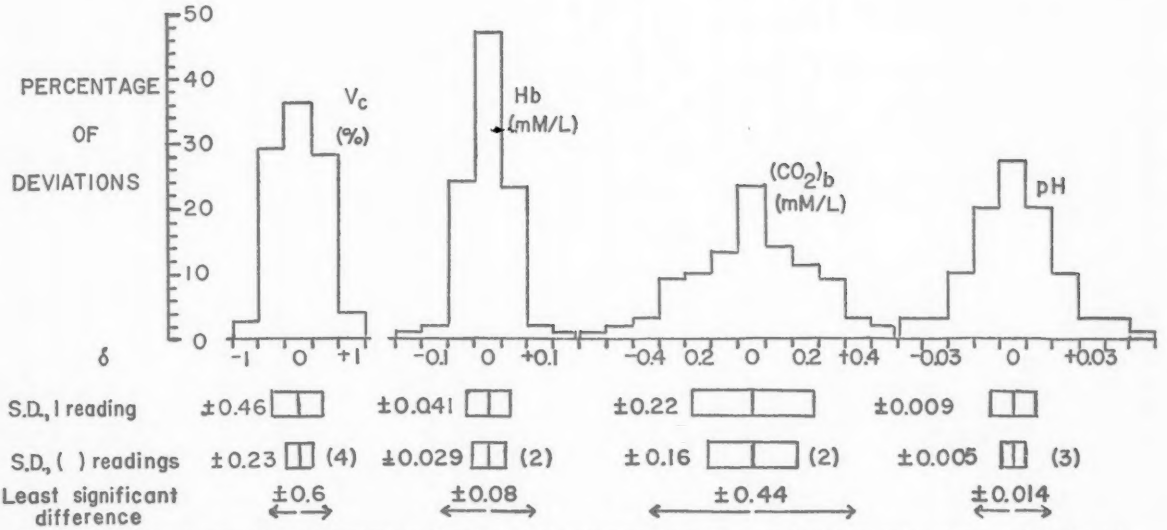

Fig. 3. Frequency diagram showing percentage distribution of deviations from the mean in a large series of sets of replicate determinations utilizing the microtechnic. Below this distribution for each variable are given the standard deviation for a single reading, the standard error of the mean of the usual number of replicates in a single determination, and the least significant difference between two determinations (at the 95 per cent confidence level).

Table 4. TEST OF COLLECTION: DROPWISE TRANSFER OF BLOOD

Specimen	*Hb* (mM/L.)	*Vc*	*pH at 37°*	$(CO_2)_b$ (mM/L)	*Heparin to blood* (ml./ml.)	*Transfer time* (min.)
Control[a]	8.05	0.400	7.300	24.1	0.3/20	—
A	8.00	0.400	7.295	23.8	0.05/0.95	1
B	—	0.410	7.295	23.8	No heparin	1
C	8.30	0.410	7.285	24.0	0.05/0.95	4
D	8.10	0.405	7.290	23.8	No heparin	4

[a] Venous blood, drawn and stored in a 20-ml. syringe containing 0.3 ml. heparin solution. By glass electrode the *p*H of this blood was 7.47 at 26°, or 7.315 at 37°, with temperature factor of Rosenthal, *J. Biol. Chem.*, 173, 25 (1948).

tained no heparin; all syringes contained some mercury for mixing the blood in the sampling process. Two of the transfers were accomplished "rapidly" (about 1 ml. of blood in 1 minute) and two "slowly" (1 ml. in 4 minutes), to simulate easy and difficult fingertip collection. As is evident from Table 4, very slight decreases were observed in *p*H and CO_2 content in the transferred blood as compared with the control blood. In syringe C the *p*H decrease of 0.015 was barely significant; all other differences in *p*H and CO_2 content were smaller than the least significant differences determined above. The factor of dilution of blood with heparin saline introduces an additional uncertainty in the measurement of cell volume and hemoglobin and CO_2 concentrations. This may explain the rather high hemoglobin concentration in Sample C (wetting of the syringe surfaces might reduce the effective amount of heparin saline dilution of the blood, in some instances). This experiment also shows that the heparin solution has had no significant effect on the *p*H of the blood to which it was added. Other experiments have confirmed this lack of effect, even when relatively much larger amounts of heparin solution were used.

Table 5. "VIRTUAL" ANAEROBIC TECHNIC: TRANSFER OF BLOOD FROM SYRINGE TO CENTRIFUGE TUBE

Method of transfer	*pH at 37°*	$(CO_2)_b$ *(in blood)* (mM/L.)	$(CO_2)_p$ *(in plasma)* (mM/L.)
Anaerobic control—syringe to syringe	7.34	24.30	28.60
"Virtual" anaerobic—Single transfer[a]	7.34	24.25	28.45
"Virtual" anaerobic—5 transfers[a]	7.32	24.00	28.10
Difference per transfer[a]			
Single transfer	0.00	−0.05	−0.15
Multiple transfers	−0.004	−0.06	−0.10

[a] Venous blood, exposed briefly to air during transfer from syringe to small centrifuge tube and back again, after stoppering tube so as to exclude air. Mixing of blood carried out with mercury. Blood heparinized in first syringe.

In another experiment we observed the effect of transferring about 8 ml. of blood from a syringe into an open centrifuge tube (a Pyrex round-bottom tube, 1.25 by 10 cm.). The blood was exposed to air during the filling process until a rubber stopper and needle were inserted as described previously, so as to eliminate any air bubble, and exposed again when the sample was taken up into a second syringe for storage. A portion of the blood in the syringe was then transferred aerobically to a 5-ml. centrifuge tube, stoppered so as to exclude air, centrifuged, and the plasma drawn off and stored in a third syringe for analysis of plasma CO_2 content. The *p*H and the whole blood CO_2 content were determined on the blood in the second syringe, and control analyses performed on the original blood not subject to aerobic transfer. The results are shown in Table 5. In addition to the single tube transfer (as described above) the experiment was repeated with 5 successive transfers to separate tubes before the blood was drawn up in the storage syringe. The differences observed as a result of this multiple transfer were divided by 5, to give figures which should be a more accurate approximation of the differences due to a single transfer. Per single transfer the *p*H was found to decrease 0.004 (an amount too small to be detected in the single transfer), the whole blood CO_2 content decreased about 0.06 mM/L., and the plasma CO_2 content, 0.10 mM/L. These changes are well within the confidence limits found for the microtechnic and we can conclude that this method of aerobic transfer of blood for centrifugation introduced no appreciable error for clinical and most other purposes.

Comparison of Arterial and Cutaneous Blood Samples

Results are presented in Table 6 for the analysis of seven pairs of blood samples, one arterial and one cutaneous in each pair. The arterial sample was drawn in a 10-ml. syringe to which 0.3 ml. of heparin solution had previously been added. The syringe was attached to a manifold connected with a needle placed in the femoral artery. Because of risk of clotting in the long connecting tube of fine bore it was generally not possible to prolong collection of the arterial sample to correspond exactly with the time required for collection of the cutaneous sample. The average time for the latter was 2.0 minutes, and for the former about 0.5 minute. Slight mean differences were observed in packed cell volume and *p*H, and virtually none in CO_2 content. The only significant mean difference was that for hemoglobin, which was 0.19 mM/L. higher in cutaneous than in arterial blood. Although significant, this difference is small enough to ignore for most acid-base analyses, in which cutaneous blood can be considered almost identical with arterial blood.

Table 6. SIMULTANEOUS ARTERIAL AND CUTANEOUS BLOOD SAMPLES
Comparison of Acid-Base Properties

	Arterial	*Cutaneous*	*Difference*
Hemoglobin (mM/L.)	8.50	8.60	−0.10
	7.80	8.05	−0.25
	8.45	8.60	−0.15
	8.20	8.20	0.00
	6.65	7.00	−0.35
	6.65	7.05	−0.40
	7.85	7.85	0.00
Mean			−0.19
S.E.			±0.064
*p*H	7.41	7.41	0.00
	7.53	7.55	−0.02
	7.35	7.35	0.00
	7.45	7.46	−0.01
	7.39	7.37	+0.02
	7.52	7.52	0.00
	7.33	7.35	−0.02
Mean			−0.004
S.E.			±0.0053
Red cell volume (mM/L.)	0.425	0.435	−0.010
	0.400	0.415	−0.015
	0.420	0.420	0.000
	0.395	0.395	0.000
	—	—	—
	0.345	0.365	−0.020
	0.400	0.395	+0.005
Mean			−0.007
S.E.			±0.0058
Whole blood CO_2 (mM/L)	23.3	23.6	−0.3
	32.2	32.4	−0.2
	21.3	21.3	0.0
	25.7	25.6	+0.1
	21.0	21.6	−0.6
	26.4	26.0	+0.4
	21.6	21.1	+0.5
Mean			−0.01
S.E.			±0.147

DISCUSSION

The suitability of this microtechnic for clinical studies of acid-base balance will be discussed from three aspects: the use of cutaneous blood; errors of collection and handling; and analytical accuracy.

Advantages of Cutaneous Blood

Our results confirm those of Lundsgaard and Møller (18), Saito (19) and Lilienthal and Riley (20) that cutaneous blood (drawn from hyperemic skin) is nearly identical with arterial blood in its acid-base properties. A comparable similarity has also been demonstrated for oxygen saturation (3, 18, 19). The only significant difference that we found was a slightly higher hemoglobin concentration, 0.2 mM/L. greater in cutaneous than in arterial blood. Part of the difference may have been due to dilution error but, regardless of the cause, the difference has a negligible effect on the use of this parameter in the calculation of CO_2 pressure and whole-blood buffer base concentration. Such a similarity of chemical properties is a fortunate circumstance for clinical studies, because the sampling of cutaneous blood is a much simpler process than an arterial puncture. The advantage of ease of sampling is thus combined with the further advantage of having arterial or cutaneous blood, rather than venous blood, for analysis of the state of acid-base balance of the patient. The respiratory factor in this state is most conveniently defined by the alveolar, or its equivalent, the arterial, CO_2 pressure, which can be readily calculated from the *p*H and CO_2 concentration of arterial blood or plasma. The usual arterio-venous differences with regard to O_2 saturation, CO_2 pressure, *p*H, and CO_2 content are greatly influenced by unpredictable variations in local venous flow. Consequently venous blood has the disadvantages of a wider range for "normal" values, a CO_2 pressure higher than in alveolar air, and a variable O_2 saturation, which introduces an additional factor in the calculation of P_{CO_2} and buffer base (14, 16). All these drawbacks are avoided by the simple expedient of using cutaneous blood for acid-base studies.

Collection, Handling, and Storage

The method described in this paper for the collection, handling, and storage of cutaneous blood samples is based on previous work (3, 21) and has here been shown to produce essentially no change in *p*H and CO_2 pressure, and only an insignificant decrease in bicarbonate and buffer base concentration amounting to less than 0.2 mEq./L. The anticipated effects of exposing blood to room air are a fall in P_{CO_2} and bicarbonate, a rise in *p*H, and no change in buffer base. However, a variety of effects may be produced by other sources of error, such as temperature change, the presence of an anticoagulant, glycolysis, hemolysis, and change in permeability of the red cell membrane. The combined error of a given method of handling blood therefore depends on a number of factors in addition

to exposure of the blood to air; in this method the results suggest a combination of slight accumulation of fixed acid, such as lactate, in the blood, and loss of displaced bicarbonate. The fact of importance in clinical chemistry is that the combined handling error is negligible if (1) heparin is used as anticoagulant, (2) the blood is chilled and stored in a closed syringe, and (3) exposure to air during collection or transfer is restricted to the conditions described herein.

Since the transient exposure to air does not introduce serious error, a technic of this sort might be called a "virtual anaerobic" technic. Undoubtedly the best method of avoiding this type of error is the "strict anaerobic" technic of Austin *et al.* (22), in which blood is handled in tonometers over mercury. However, these precautions are scarcely feasible or necessary in clinical studies of acid-base balance. On the other hand, such a "virtual anaerobic" technic appears to offer definite advantages over the common practice, in hospital laboratories, of relying on the sometimes uncertain protection (23–25) afforded by handling blood under oil, or of adhering to the outmoded "plasma CO_2 combining power" method (26), which was abandoned in Van Slyke's laboratory more than 30 years ago (27). Centrifuging blood under oil in the ordinary centrifuge tube is almost certain to produce appreciable loss of CO_2 (23), whereas use of a syringe (21) or a completely filled tube entails no appreciable loss (23; Table 6). Even with 1-ml. samples it is a convenience to avoid having to sample blood or plasma under oil; and we have demonstrated a definite decrease in accuracy of measuring 0.1 ml. aliquots when this is done from a sample under oil.

A certain amount of training is required for the analyst to attain proficiency in the collection of cutaneous blood samples and in the filling of the micropipets. The experience gained in acquiring such proficiency and in teaching others leads us to believe that the method is entirely suitable for any hospital laboratory where one or more individuals can be trained in its use, under the supervision of a clinical chemist.

Accuracy

The accuracy of the individual determinations is satisfactory for all clinical and many experimental purposes, as shown by the results in Fig. 3, and various papers describing each method (1, 2, 7, 28). The standard errors of measurement shown in Fig. 3 are estimates of reproducibility rather than of absolute error, which may be somewhat larger. For example, the hematocrit method is subject to errors due to trapping of solution between cells and to anisotonicity of the 0.9% NaCl diluent with respect

Table 7. Comparison of Colorimetric and Potentiometric pH in Human Plasma at 38°
Data from the Literature

Source	No. comparisons	Difference, $pH_{col} - pH_{pot}$		Remarks
		Mean	S.D.	
Hastings and Sendroy[a] 1924 (6)	5	+0.007	±0.011	3 comparisons at 20°. In vitro equilibration.
Myers and Muntwyler 1928 (38)	41	+0.020	±0.037	Venous plasma, as drawn from patients. Microcolorimeter for *p*H.
Fosbinder and Schoonover 1930 (39)	28	+0.002	±0.011	Plasma diluted 1–20 with KCl-phenol red and equilibrated in vitro.
Austin, Stadie, and Robinson 1925 (36)	2	+0.035	±0.015	One comparison with *p*H calculated from Henderson-Hasselbalch equation.
Van Slyke, Weisiger, and Van Slyke 1949 (2)	26	−0.002	±0.022	Equilibrated in vitro, *p*H range 6.89 to 7.65. Photocolorimetric *p*H.
Weighted Mean	102	+0.008	..	

[a] Hastings and Sendroy found a difference of −0.015 ± 0.013 in 13 comparisons in horse plasma, and −0.010 ± 0.009 in 4 comparisons in dog plasma.

to the actual red cell contents in a given blood. This uncertainty is not important in the calculation of acid-base factors, but of the two parameters, the hemoglobin concentration is a more accurate determination than the hematocrit value. The precision of the manometric blood-gas apparatus is well established for this as well as macro determinations (1, 28, 29). Unsuspected errors have been shown to be far greater in other methods commonly used in clinical laboratories (30), and best results cannot be obtained without careful attention to detail and accurate calibration of photometer tubes and pipets.[7] The methods described in this paper have been successfully employed in a number of clinical studies (32–34).

The only part of the microtechnic to which objections have been raised in the past is the colorimetric *p*H determination. In comparison with the electrometric method (H_2 or glass electrode), the colorimetric method has been charged with being unreliable when applied to plasma or serum

[7] Stock volumetric pipets as purchased may deviate considerably from the stated volume and this is particularly true of the 20-cu.mm. hemoglobin pipets (31). In our experience the individually tested hemoglobin pipets supplied by Arthur H. Thomas Co. have been quite satisfactory; in one batch of 24 pipets calibration showed a maximum deviation from the 20-cu.mm. volume of only 0.3 cu.mm.

(35–37), and this point deserves comment. Large discrepancies found have been ascribed to variability in the effects of dilution, protein concentration, and other errors on the final result. When available reports of comparisons between electrometric and colorimetric *p*H are examined, it becomes evident that details of procedure have varied widely. Some comparisons have been made with dog instead of human blood, with serum instead of plasma, with the colorimetric *p*H read at room temperature instead of 38°. The only comparisons of relevance here are those made following the basic Hastings and Sendroy technic, on which the Shock and Hastings and current modifications are based. A summary of relevant studies of this type on human plasma is given in Table 7. Results are in terms of the difference between the colorimetric and potentiometric *p*H, both determined at 38°. Most of the papers do not present figures for the mean and standard deviation, which we have calculated from the data for the individual differences. It is evident that there is generally good agreement in the several series, and the weighted mean difference is less than +0.01 *p*H unit. If the correction in Table 2 is applied, assuming an average comparison *p*H of 7.40, the mean difference is seen to fall between 0.00 and −0.01. The reproducibility of the difference, as adjudged by the generally small standard deviation, is good. In a series of 15 comparisons of glass electrode with H_2 electrode *p*H, Van Slyke *et al.* found a mean difference of −0.002 and a standard deviation of ±0.016 (2), a reproducibility only slightly better than for the photometric *p*H. On the basis of these result it would appear that the difference between the photocolorimetric and potentiometric *p*H determinations at 38° lies between −0.04 and +0.04 in over 90 per cent of cases.

The discrepancies referred to previously have not been reported on human plasma, but generally on dog serum, and in connection with variability of the "C" correction, the difference between the colorimetric *p*H at 20° and photometric *p*H at 38°. Species differences and differences between serum and plasma with respect to the "C" correction have been observed by Cullen and many others (35, 36, 40–42). The "C" correction is +0.30 for human serum and +0.23 for human plasma, and Robinson *et al.* demonstrated that the correction varied with the serum protein concentration (43). Some of the results on dog and human serum have been characterized by large standard deviations, ranging from ±0.07 to ±0.11 (35–37). On the other hand, Hastings and Sendroy (6) found little variation from the plasma of one species to another when the colorimetric-potentiometric difference was measured at 38°. The same is true of this difference in human serum as compared with plasma, at 38°

(38, 41). From all this and other evidence (44) it seems clear that the final result in a colorimetric *p*H determination is influenced by a number of possible errors, which are generally larger when serum is used or when the reading is made at room temperature. For human plasma read at 37–38°, these errors either become very small or are self-compensating (44), as is demonstrated by the evidence in Table 7.

In their practical operation for the determination of *p*H in blood, both the H_2 electrode and glass electrode have serious disadvantages that must be weighed along with their advantages, in comparing them with the photocolorimetric method. For clinical work economy of sample size is often an important consideration, and in this the photometric method is superior to the usual glass electrode. The apparatus required for the photometric determination is cheaper and probably more reliable than the glass electrode and *p*H meter, but the technic of the determination is somewhat more complex. The standard error of measurement in the photometric *p*H determination appears to be *smaller* than that for any commercially available *p*H meter utilizing the glass electrode. After experience with both methods our preference in clinical work is definitely for the photometric.

Some of the features of the "virtual anaerobic" technic described here are applicable to the handling and centrifuging of larger amounts of blood, 2–20 ml., for the determination of *p*H and CO_2 concentration by other methods.

SUMMARY

1. A modification has been described for the Shock and Hastings technic of determining simultaneously, in 0.1 ml. aliquots of blood, the *p*H, CO_2 content, and packed cell volume. Advantages of the modification are collection and handling of the blood in a way that avoids the use of oil, and a modified design of the micropipets so that *p*H readings can be obtained in a filter photometer.

2. The standard error of measurement of a single reading was ± 0.009 for the *p*H, and ± 0.22 mM/L. for the whole blood CO_2 concentration. The reproducibility of the photocolorimetric *p*H measurement was slightly better than that obtainable with the most accurate commercially available glass electrode.

3. Results have been presented to show that blood can be handled in the presence of brief, limited, exposure to air with no detectable change in *p*H and only negligible change in CO_2 content (decrease of less than 0.2 mM/L.). Errors attributable to collection and handling of cutaneous blood by this "virtual anaerobic" technic are not of significant magnitude.

4. Results have also been presented in confirmation of the fact that arterial and cutaneous blood are virtually identical in acid-base properties. A sample of as little as 0.5 ml. of cutaneous blood, analyzed by the microtechnic, provides the necessary and sufficient data for a description of the state of acid-base balance. This includes calculation of the physiologically important variables, arterial CO_2 pressure, and whole-blood buffer base concentration, thus furnishing a measure of the respiratory factor and the metabolic factor in any acid-base disturbance. With a sample size of 1.5 ml. it is also possible to determine other individual constituents of plasma, such as sodium, chloride, and total protein.

5. The technic is useful in the study of clinical disturbances of acid-base balance, especially in infants and children.

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Estimation of Lipid Fractions of Blood Plasma

System Using the Allen Volumetric Method

Andre C. Kibrick and S. J. Skupp

ABNORMALITIES IN THE VALUES of the lipid fractions of blood plasma, except for cholesterol, are not generally employed as a guide in following various clinical conditions, since the laboratory procedures available for their estimation have been quite cumbersome. This deficiency in convenient methods for determining the various lipids of blood is particularly unfortunate at this time when one considers the important role that the various lipids may play in such conditions as atherosclerosis and heart disease (1). In 1934 Allen devised a volumetric method for estimating blood fat of cattle which involved final measurement of the column of fat in a graduated capillary tube (2). Although such volumetric methods were applied to clinical chemistry at about that time (3), they have since been discarded for the most part.

We have recently reinvestigated the reliability of the Allen method on human plasma. By calibrating the tubes for recovery of known amounts of fat instead of using the content of the capillaries, we have obtained satisfactory values for the total of free cholesterol, esterified cholesterol, and neutral fat—that is, for all the lipid fractions except phospholipids which are not determined by this procedure. In the present paper we

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present data on the reliability of the Allen method and describe its application to a simple system for determining the major lipid fractions of blood plasma.

ADAPTATION OF ALLEN METHOD

The method for the volumetric determination of blood fat was performed essentially as described by Allen (2) on 3-ml. samples of plasma. The 2-neck tubes[1] were the same, and carried graduated capillaries in which the column of fat could be measured with calipers on the millimeter graduations. The samples were measured into the tubes by applying gentle suction to the capillary necks. A 5-ml. amount of reagent[2] was then added in the same way and mixed thoroughly with the plasma by rotation. The unstoppered tubes were heated in a water bath at 85° for 20 minutes with occasional shaking. They were then centrifuged for 5 minutes at 800 rpm., and warm water (75°) was added with a hypodermic needle attached to an elevated reservoir until the tuber were filled to the bottom of the graduated neck. After centrifuging again for 2 minutes, warm water was added until the filling neck overflowed and automatically brought the fat column to the proper level for reading. The tubes were again centrifuged for 5 minutes and placed in a 60° water bath for a few minutes before measuring the fat column with calipers.

The tubes were initially calibrated with mercury according to standard procedure, and the values obtained agreed precisely with the factors obtained by titration of the amount of standard acid contained in the capillaries with standard alkali. However, when the Allen method was performed on known amounts of olive oil, more than 100 per cent recovery of the oil was obtained by using the calibration factors. This was true when the oil was measured into the tubes by taking an aliquot of an ether solution and the ether then evaporated off, or when the oil was carefully weighed on a microbalance and added without solvent. In about 50 experiments the values of olive oil recovered usually ranged between 122 and 129 per cent. An average value of 25 per cent was therefore used to correct the mercury calibration factors.

The validity of the calibration values derived from the recovery of olive oil was checked in several ways. There was no indication of contamination

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[1] Allen tubes for 3 ml. and for 0.25 ml. of plasma were obtained from E. Machlett and Son, New York. The micro tubes for 0.25 ml. are still being tested and will be reported in a later publication.

[2] The reagent consists of 110 Gm. sodium carbonate and 200 Gm. sodium salicylate dissolved in water and made up to a volume of 1 liter. To this are then added successively 30 ml. of 50% sodium hydroxide and 100 ml. of *n*-butyl alcohol.

of the fat layer with constituents of the Allen reagent. Neither butyl alcohol nor salicylate was found as a contaminant, when the recovered fat was tested with ammonium molybdate or ferric nitrate. The absence of significant amounts of either of these constituents of the reagent was confirmed by the fact that the iodine numbers of olive oil and other fats and oils were exactly the same on samples of the fat layer obtained after performing the Allen method (4) as when the original oil and fat were used as samples. The iodine number of butyl alcohol and sodium salicylate is 0 and 99, respectively, which differ quite markedly from that of olive oil and some of the other fats and oils. Since the values of the iodine number are the same before and after separation by this procedure, the method appears to separate oil and fat in the same degree of purity as the original lipid samples.

Since the apparent volume of the fat layer is larger than that of the original weight of the oil taken, we are forced to the conclusion that the volumes of oil measured in capillaries of this size are affected by spreading factors that may be unique for oils and fats (5). This conclusion is consistent with the results obtained by Chung, Saarinin, and Shaw (6) with the Allen method.

We have confirmed the original findings of Allen that the fat layer contains all the lipids of plasma with the exception of the phospholipids. In many determinations of the fat layer for phosphorus, not more than 5–7 per cent of the phosphorus of the phospholipids of the plasma was ever found. In addition, no fat was separated when the Allen method was performed on lecithin. The cholesterol of plasma was always quantitatively recovered in the fat layer, and, as mentioned above, neutral fat and oils are also quantitatively recovered. In the case of the fatty acids esterified with cholesterol we had more trouble. It was considered quite possible that the mild alkaline hydrolysis which is part of the Allen procedure would liberate the fatty acids, and we have found that free fatty acids are not contained in the fat layer. This point was finally resolved when we found the same amount of both total and esterified cholesterol in the fat layer of plasma as was present in the plasma itself.

The Allen method is conducted essentially as described by Allen with either 3 ml. or 2 ml. of plasma, and the calibration is made as described above, based on recovery of olive oil. The only other change in the method is that the hydrolysis and entire procedure are conducted in open tubes, which eliminates the necessity of using toothpicks to stopper the tubes.

Besides the Allen method, our system of analysis comprises the determination of cholesterol and cholesterol esters in plasma (7) and the determination of lipid phosphorus (8). From the values so obtained the following calculations are made.

CALCULATIONS

1. Lipid P $\times$ 25 = phospholipids
2. Esterified cholesterol $\times \frac{277}{386}$ (9) = cholesterol fatty acids
3. Allen fat $-$ cholesterol $-$ cholesterol fatty acids = neutral fat
4. Allen fat $+$ phospholipids = total lipids
5. $\frac{831}{869} \times$ neutral fat $+$ cholesterol fatty acids $+$ 0.64 phospholipids = total fatty acids

RESULTS

Total Fatty Acids

In Table 1 we compare the values of total fatty acids found by these calculations in a representative series of plasma samples with values of the total fatty acids obtained by the colorimetric method with dichromate (10) and by the iron perchlorate method for esterified fatty acids (11).

The values of the various lipid fractions found by this system of analysis agree in general with values found in the literature. In several samples we oxidized the total unsaponified lipid residue of plasma by the method of Bloor (12) with dichromate and calculated the values of the various fractions by using their respective factors in milliliters of dichromate. The values agreed quite well with those obtained by our system of analysis. This is especially encouraging in the case of the neutral fat since this fraction has been most neglected in the literature and since it is the least reliable due to the fact that it is always measured by difference.

Neutral Fats

We show in Table 2 the values of neutral fat found in duplicate by our method in 14 apparently healthy normal individuals. It is interesting that all of the values except for Number 8 fall close to zero. Number 8 was a man of about 45 years old, who proved to be an alcoholic and may have had some liver dysfunction. Some of the values may be seen to be slightly negative and must be considered zero. Considering the good agreement between the results of duplicate determinations, the slightly negative values in the table are an indication of the sensitivity of the method with respect to the calculated value for neutral fat.

Table 1. TOTAL FATTY ACIDS IN PLASMA

Comparison Between Values Determined by the Present System and Those Determined by the Colorimetric Dichromate and Iron Perchlorate Methods

Sample	*Total fatty acids*	
	Allen (mg./100 ml.)	*Dichromate* (mg./100 ml.)
1	373	402
2	322	328
3	309	300
4	323	350
5	309	336
6	373	396
7	422	425
8	317	313
9	309	291
10	421	441
11	313	343
12	304	343
13	392	392
14	264	274
15	297	296
16	309	320
17	268	282
18	172	189
19	281	264
20	297	296
21	307	315
22	177	197
23	386	378
24	265	269

Table 2. VALUES OF NEUTRAL FAT IN THE PLASMA OF 14 NORMAL INDIVIDUALS

Subject #	*Neutral fat* (mg./100 ml.)
1	3, 4
2	18, 35
3	47, 65
4	24, 38
5	−17, 2
6	13, 25
7	13, 11
8	142, 123
9	133, 88
10	88, 88
11	−12, −9
12	11, 36
13	13, 15
14	−14, 0

Values are given for duplicate determinations.

DISCUSSION

The advantage of the described system of analysis is its simplicity. Values of neutral fat are calculated with a reasonable degree of validity, and the other fractions are estimated by standard methods. The simplicity and lack of technical difficulty of our method are not so true of other methods. Although total fatty acids may be determined colorimetrically in plasma quite readily by oxidation with dichromate (9), the estimation of other lipid fractions by dichromate oxidation is still difficult technically and involves uncertainties due to calculation from the dichromate factors of the various fractions. The determination of total fatty acids of plasma is also possible by the method of Bauer and Hirsch as a measure of the esterified fatty acids (10). Although this method is reliable when all factors are carefully controlled, very small differences in the time in which reagents are added at each step seriously affect the results. In addition, the method yields only the total fatty acids and can not be used to estimate the other lipid fractions.

SUMMARY

The Allen volumetric method for plasma fat has been modified by calibration to include the percentage of olive-oil recovery. The values obtained from calibration with mercury are corrected for the 125 per cent recovery of the oil. The validity of the entire procedure has been discussed.

A simple system of estimating the lipid fractions of plasma has been described. Total lipids are the total of Allen fat + (25 × lipid P). Neutral fat is Allen fat-cholesterol-cholesterol fatty acids. Total fatty acids are $\frac{831}{869}$ × neutral fat + cholesterol fatty acids + (0.64 × phospholipids).

The values of total fatty acids have been compared with those obtained by oxidation with dichromate and by determining the esterified fatty acids with iron perchlorate.

The values of neutral fat found in 13 apparently normal individuals are shown to range from 0 to 88 and to give an average value of 28 mg./100 ml.

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Iodine and Thiocyanogen Numbers of the Fatty Acids of Blood Plasma

Andre C. Kibrick and S. J. Skupp

LITTLE IS KNOWN about the degree of unsaturation of the fatty acids of human blood (1). A spectrophotometric method has recently been applied to plasma by Wiese and Hansen (2) which consists of conjugation of the double bonds by heating in alkaline solution at 180° and measuring the conjugated forms in the ultraviolet spectrum region. The authors themselves admit that this procedure is subject to much uncertainty since standards are not available for fatty acids with more unsaturated bonds than arachidonic acid. The application of this time-consuming method in clinical chemistry would, therefore, not be warranted at this time.

In order to study the degree of unsaturation of the fatty acids of blood we have chosen, therefore, to use the values of iodine and thiocyanogen numbers, which have proven useful in the analysis of oils and fats (3). In 1931 Yasuda (4) proposed the use of the Rosenmund and Kuhnhenn reagent (5) to determine the iodine number of the fatty acids analyzed according to Bloor (6). We have also found that the iodine number of the fatty acids of blood plasma may be determined in this way, but have improved the oxidative method of Bloor for colorimetric estimation of the fatty acids (7). However, we have found that the thiocyanogen number (8) can not be determined in fatty acid extracts prepared by the method of Bloor. In the present paper, we describe a method to determine both iodine number and thiocyanogen number of some of the fatty

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acids of blood plasma by employing the fat isolated mechanically by the volumetric method of Allen (9).

METHOD

Reagents

1. Allen reagent (9).
2. Rosenmund-Kuhnhenn reagent (7) for iodine number.
3. Thiocyanogen reagent (8).
4. 0.1N sodium thiosulfate.
5. Potassium iodide, 1 %. Prepare as needed.

Fat is isolated in Allen tubes from 3 ml. of plasma by the method of Allen as described by the present workers. The fat column is measured in millimeters and calculated as fat content from calibration constants obtained in the usual manner with mercury. The experimental values are also corrected for the percentage recovery of known amounts of olive oil (10). The tubes are then returned to the 60° water bath. Fat is removed with 23-gage 3–4-inch hypodermic needles attached to a syringe, and about 5 mg. are weighed in microcombustion boats on a micro or semimicro balance. The boats containing the fat are then placed in 50 ml glass-stoppered Erlenmeyer flasks. For iodine numbers, 2 ml. of the Rosenmund-Kuhnhenn reagent and 3 ml. of chloroform are added. After mixing, the flasks are allowed to stand for 15 minutes in the dark. The excess reagent is then titrated with 0.1N thiosulfate, using a micro buret, after the addition of 0.5 ml. of 1 % potassium iodide.[1] For thiocyanogen numbers, 2 ml. of reagent and 3 ml. of carbon tetrachloride are added, and the mixture is allowed to stand for 48 hours in a dark water-cooled cabinet at 18–20°. The excess reagent is then titrated with 0.1N thiosulfate. In each case reagent blanks which do not contain fat are titrated in the same way. Since the cholesterol[2] in the isolated Allen fat absorbs iodine, its concentration in the plasma samples must be determined by a reliable method (11).

Calculation

$$(\text{Blank titer} - \text{Unknown titer}) \times 12.69 = \text{mg. iodine absorbed by fat.}$$

$$\frac{\text{Wt. of fat in titration flask}}{\text{mg. Allen fat/100 ml. plasma}} \times \text{mg. cholesterol/100 ml. plasma} = \text{mg. cholesterol in flask.}$$

[1] Iodine numbers have also been determined in the Allen fat with the Wijs reagent.

[2] Experimental iodine number of cholesterol = 0.69. Experimental thiocyanogen number of cholesterol = 0.12.

$$\frac{\text{mg. iodine absorbed} - (0.69 \text{ mg. chol.}^2 \text{ in flask})}{\text{mg. fat in flask}} \times 100 = \text{Iodine number}$$

$$\frac{\text{mg. iodine absorbed} - (0.12 \text{ mg. chol.}^2 \text{ in flask})}{\text{mg. fat in flask}} \times 100 = \text{Thiocyanogen number}$$

The method was checked on olive oil and other fats and oils and was found to give the same results as both macro and micro determinations on samples of the fats or oils before going through the Allen procedure. We also determined simultaneously the iodine number of a large number

Table 1. Comparison Between the Values of Iodine Number Determined by the Present Method and the Yasuda Method

Plasma sample #	*Iodine number*	
	Present method	*Yasuda method*
1	107	107
2	100	110
3	104	102
4	123	111
5	103	97
6	108	102
7	109	101
8	102	106
9	120	115
10	105	113
11	102	106
12	109	101

Table 2. Values of the Iodine and Thiocyanogen Numbers Determined in 14 Normal Individuals

Subject #	*Iodine number*	*Thiocyanogen number*	*Difference*
1	123	107	16
2	132	76	56
3	151	73	78
4	133	92	41
5	111	64	47
6	127	102	25
7	134	86	48
8	93	71	22
9	124	111	13
10	116	64	52
11	127	118	9
12	138	90	48
13	157	122	35
14	128	91	37

[2] Experimental iodine number of cholesterol = 0.69. Experimental thiocyanogen number of cholesterol = 0.12.

of plasma samples by this method and by the method of Yasuda. Table 1 shows some representative results. It is seen that the present method yields values not much different from those obtained by the Yasuda method in spite of the absence of the fatty acids of the phospholipids in the Allen fat.

In Table 2 we show the results of iodine and thiocyanogen numbers obtained by our method on the fat isolated from plasma in 14 supposedly healthy laboratory workers.

DISCUSSION

The iodine number, which represents the average degree of unsaturation of the fatty acids, might be expected to be affected in liver dysfunction since there is considerable evidence that the liver is the major site of desaturation (12). Wachstein has found high values in parenchymatous or obstructive jaundice (13), and Nicholls and Perlzweig have found high values in hyperthyroidism (14), a condition in which fat metabolism may be affected.

It was considered likely that a knowledge of both the iodine and thiocyanogen numbers in the same lipid fraction would yield more information about the desaturation mechanism. The iodine number is a measure of the total number of unsaturated bonds of the fatty acids, whereas the thiocyanogen number apparently measures the number of these bonds in acids containing only one unsaturated linkage and one less than the total number of unsaturated bonds in acids containing two or more (3). Oleic acid may be present in considerable amount in blood and tissues. We have found about 50 per cent of the fatty acids to be unsaturated and about 25 per cent to contain more than one unsaturated bond by other methods (15). Since linoleic and linolenic acids are present in only a small amount in normal rat tissues (16), this indicates an appreciable content of other unsaturated acids. Hence, the simple calculation from the values of the iodine and thiocyanogen numbers that can be made for commercial oils (8) are not possible for biologic material of this type. However, the difference between the values of the two numbers reflects the content of fatty acids containing more than two unsaturated bonds.

It is seen in Table 2 that the iodine numbers in our small series of normals range from 111 to 157. Number 8 may be excluded since it represents an adult male of about 45 who was later found to be an alcoholic and to have other indications of liver damage. The differences between the values of the iodine and thiocyanogen numbers found in our small series of normals are rather disappointing. The presence of

both small and large relative amounts of fatty acids containing more than two unsaturated bonds in this lipid fraction indicates that it has little significance in disease. The difference between the two numbers may well be a reflection of the diet.

This investigation is being continued to learn the significance of the values of the iodine number in liver disease and in other clinical conditions in which there is a disturbance of fat metabolism. We are also endeavoring to improve the present procedure to yield the iodine and thiocyanogen numbers of the total fatty acids of plasma and of the fatty acids of each lipid fraction.

SUMMARY

A method has been described for the determination of both iodine and thiocyanogen numbers of the fat isolated from human blood plasma by the Allen volumetric method. The values found in 13 normal individuals varied from 111 to 157 for iodine number and from 64 to 122 for thiocyanogen number. The differences between the two numbers varied between 9 and 78.

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The Correspondence with Beer's Law for the Optical Density of Stained Protein Patterns on Filter Paper as a Function of Surface Protein Concentration

V. H. Rees and D. J. R. Laurence

THE SCANNING TECHNIC for evaluating data from electrophoresis on filter paper, which was first described by Grassmann, Hannig, and Knedel (1) and by Eisenreich and Eder (2), has now become established as routine procedure in many laboratories. Although many types of scanning have been described, they have in common the advantage that the colorimetric determinations are made on the paper strip upon which the protein pattern has been developed. The difficulties of quantitative elution of the colors are thereby avoided. Crook, Harris, and Warren (3, 4) have emphasized that the simple Beer's logarithmic law which is obtained in homogeneous solutions cannot be assumed to hold for the paper matrix without detailed experimental justification, and they have proposed an alternative relationship for such data. The problem of quantitation has also received the attention of many other workers, and several, notably Griffiths (5), Latner (6), Grassmann and Hannig (7), Sommerfeldt (8), and Cooper and Mandel (9) have found the application of Beer's law to be justified experimentally over the working range of the method, as long as transmission densitometry is used.

The verification of Beer's law has now become of especial interest since Laurence (10) and Cooper and Mandel (9) have described the use of a light-sensitive circuit with an output linear with the optical density. This circuit permits the automatic recording of paper electrophoretic patterns to give a tracing from which proportions of the separate fractions

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can be estimated directly by means of a planimeter, as long as Beer's law can be assumed. The value of automatic recording would be greatly reduced if the results require rectification afterwards by application of a correction curve; also the scale of the recording could no longer be adjusted electronically without restriction, if deviations from the logarithmic law must be considered.

The comparisons made by Rees and Laurence (11) between the results of automatic recording and a fluorimetric method showed indirectly that the scanning method was satisfactory, but there remained the possibility of several errors in the scanning method leading to a correct result by compensation. A detailed investigation has therefore been made of the relationship between protein concentration and optical density of the protein-dye complex. The investigation was limited to transmission densitometry only and has no bearing on the problem of the varying dye uptakes of different protein species.

THEORETICAL CONSIDERATIONS

In this paper certain terms are used as follows:

1. The surface protein concentration is the number of micrograms of protein contained in 1 sq. cm. of the filter paper, or the corresponding incremental definition if the pattern density varies continuously.

2. The optical density is $\log_{10} (I_0/I)$ where I_0 is the incident light intensity and I is the intensity of the light emerging after absorption.

3. According to Beer's law the optical density of any region should be proportional to the surface protein concentration. The application of the law is therefore tested by plotting optical density against surface concentration of protein and the result should be a straight line through the origin.

4. Simple considerations trace most deviations from Beer's law to fluctuations in absorbing power within the light beam. Examples are the use of light of several wave lengths which are absorbed to different extents, the influence of stray light which is not absorbed at all, and the effect of scattering to produce paths of differing lengths through the absorbing region. All these effects cause the optical density observed to be less than that predicted from experiments at low densities, since the observed result is the logarithm of average light intensities, whereas the required result is an average logarithm of light intensities which is always the greater. Therefore if the optical density of the paper is plotted as ordinate against surface protein concentration as abscissa, expected deviations are a bending "downward" of the curves.

MATERIAL AND METHODS

Known amounts of protein were added along filter paper strips either (1) uniformly over known areas limited by silicone barriers (termed the "wedge method," as the developed strip resembles an optical step wedge) (Fig. 1*a*) or (2) as transverse lines (the "stripe" method) (Fig. 1*c*). Wedge methods have been used by Crook *et al.* (4) and by Latner (6) and stripe methods by Griffiths (5), by Sommerfeldt (8), and by Grassmann and Hannig (7). The strips were then stained, washed and oiled, and examined by transmission densitometry. The densitometer tracing for the wedge method is a series of flat-topped segments (Fig. 1*b*). The distance between the top and the base line was calibrated in terms of optical density and the surface area of protein on the filter paper measured by means of a planimeter. As the amount of protein is known and the distribution is uniform, the optical densities of the various segments of the wedge could be plotted against surface protein concentration (μg./cm.^{2}).

The densitometer tracing for the stripe method is a series of density contours reminiscent of the patterns obtained in a conventional separation of plasma proteins (Fig. 1*d*). The areas within these contours above the base line are obtained with a planimeter and compared with the corresponding dilutions of the protein sample.

Filter Paper Strips. Whatman No. 1 (for chromatography) in strips size 29 × 4 cm.

Protein Samples. Plasma from hospital patients diluted with 0.9% saline to 2–3% w/v and also 2% solutions of bovine serum albumin.

Silicone. Dow Corning 1107 with acetone (1:1).

Wedge Method

A filter paper strip was placed on a glass plate and 7 transverse pencil lines were drawn 2 cm. apart, commencing 8.5 cm. from one end, to form a set of 6 adjacent compartments 4 × 2 cm. transverse to the length of the paper. The silicone-acetone mixture was applied along the pencil lines from a Petri dish, using the edge of a standard histologic glass slide cut to 4 cm. in length. The filter paper was then heated for 5 minutes in an oven at 105° to fix the silicone and used within 30 minutes. The paper was held horizontally by its ends in a frame. The 6 compartments were each moistened with 0.05 ml. of 0.9% saline and allowed to dry at room temperature. A series of 6 dilutions of the protein sample (at concentrations of 1.0, 0.8, 0.6, 0.4, 0.2, 0.1, taking the original sample concentration as 1.0) were made in 0.9% saline. Exactly 0.04 ml. of these solutions

was applied, one to each of the compartments of the filter paper, using a calibrated blood pipet. The sample was applied to the center of a compartment and was sufficient in amount to ensure a thorough and even wetting of the paper. Because of the silicone barriers, the samples were confined strictly within single compartments and the edges of the protein patterns were sharp. The strip was allowed to remain uncovered at room temperature for 5 minutes and then placed in the oven at 105° for 15 minutes, still horizontally supported. The dried paper was then stained as described.

Stripe Method

A filter paper strip was damped with 0.9% saline and lightly blotted to remove excess. The strip was then placed across a wire frame consisting of 5 parallel wires 3 cm. apart stretched across a laboratory wire basket. On each wire was a dry filter paper circle (Whatman No. 1, 11 cm. diameter) sharply folded to form an inverted V. The action of the frame was to drain saline from the transverse lines of the paper strip in contact with the frame. A number of 2-fold dilutions of the protein sample in 0.9% saline were made and exactly 0.02 ml. of these dilutions were applied by means of a pipet to the paper strip centrally between the wires of the frame as transverse stripes. The effect of the moisture gradient so produced in the strip was to spread the stripes laterally, producing a gradual increase in protein concentration towards the center of the stripe. Care was taken that the stripes were evenly applied and of constant length. The pattern was allowed to become almost dry in the air and then heated at 105° for 15 minutes in the oven. The dried paper was stained as described.

Dyeing

Amidoschwarz 10B (Bayer) and azocarmine B (Gurr) were employed in dyeing.[1] Amidoschwarz was used as a saturated solution (about 1.5% w/v) in 10% acetic acid–90% methanol, and azocarmine as a 0.75% solution in 10% acetic acid–45% methanol–45% water. Staining was for 10 minutes at room temperature. The background color was washed away with 10% acetic acid–90% methanol, and in the case of amidoschwarz the further washing described by Rees and Laurence (9) was used. The strips were washed finally with methanol and dried at 105° for 5 minutes in both cases.

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[1] G. T. Gurr, Ltd., London.

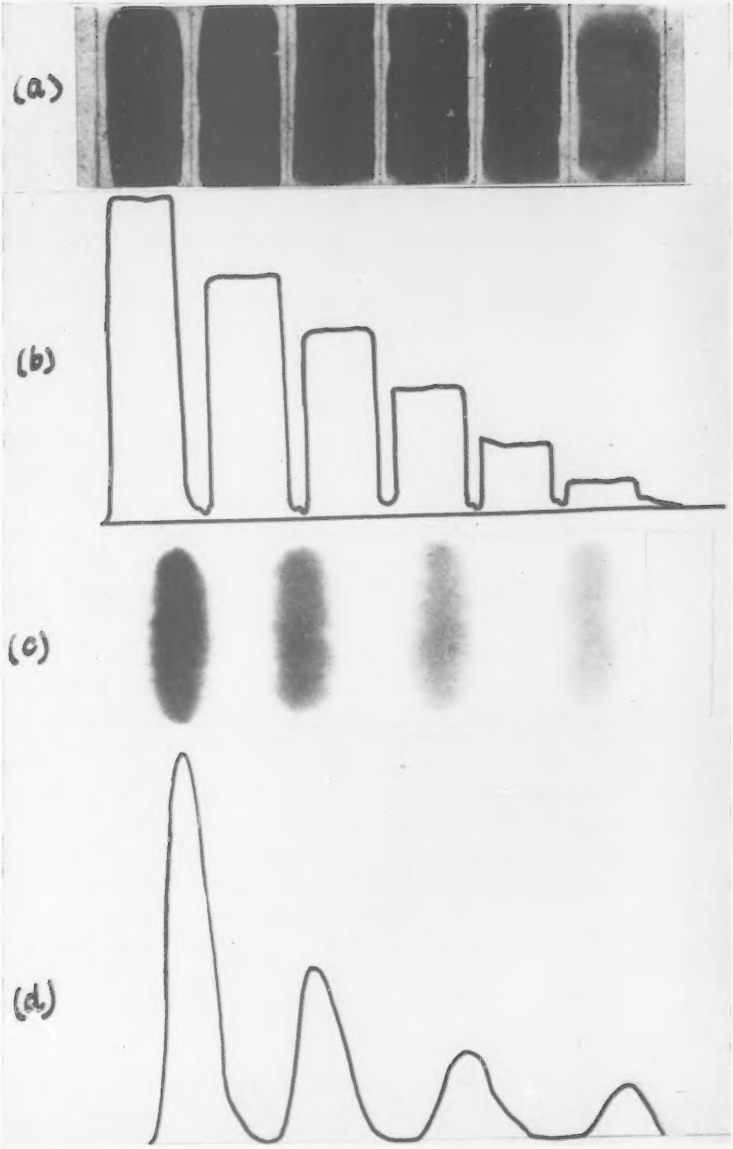

Fig. 1. Protein patterns on filter paper by the wedge and stripe methods with the corresponding densitometer tracings: (*a*) and (*b*), the wedge method; (*c*) and (*d*), the stripe method.

Oiling

The oil applied was a mixture of 1 part of α-bromonaphthalene (B.D.H.) and 2 parts of liquid paraffin (B.P.). This mixture (5 parts) was diluted with xylene (2 parts) and 1% of sorbital mono-oleate (Croda) was added to bind the oil to the paper. The paper strips were drawn through a pool of the oil on a glass plate so that the oil was taken up by the paper from the lower surface only, to avoid trapping air bubbles. The oiled paper was then slightly bowed and stood upright on a longer edge on a flat filter paper sheet to drain. After 20 minutes, most of the xylene had evaporated and the paper was lightly oiled, yet could be subject to slight pressure without exuding oil.

Scanning

The automatically recording densitometer described by Laurence (10), was used to examine the oiled papers. The paper was placed in a holder and covered with a thin glass plate to hold it flat. It was then driven between an illuminated slit and a photocell at a constant rate and the optical density was obtained from the photocell response and plotted by a Murday type recorder[2] on recorder paper moving at the same rate.

Calibration of Densitometer

The densitometer was calibrated using an Ilford[3] calibration strip on 35-mm. film, which is a neutral filter step wedge, with which an individual calibration chart in optical densities is provided by the manufacturers. In order to obtain an accurate calibration for low optical densities, the Ilford wedge was cut into 3 segments. The central segment containing a reference mark to identify it on the calibration chart was used in recording with both the higher and the lower density segments on appropriate scales of amplification. Deflections for the densest segment (M) were noted on a microammeter, which is independent of amplification used in the recording. The corresponding microammeter deflections (m) for any given segment could be obtained by measuring the deflection on the recorder chart in millimeters (d) and using Equation 1

$$m = Md/D \qquad (1$$

where D is the deflection of the densest segment in millimeters. In this way a calibraton curve of the reading of the microammeter related to optical density was obtained. This curve could be used to obtain the

[2] Evershed & Vignoles, Ltd., London.
[3] Address: London, W.C.1.

optical density scale for any given recording in subsequent work. The microammeter deflection corresponding to the densest part of a recording was noted and from this the deflections for all parts could be calculated using Equation 1. These microampere deflections could then be converted to optical densities using the calibration curve.

Specifications of the Densitometer

The following characteristics of the recording densitometer must be mentioned in the present context. The light source is a 6W, 6V festoon lamp with a linear filament about 1 cm. long, and the light from this filament converges on the slit 2 cm. × 0.5 mm. through a double plano-convex molded condenser lens system. The lens system is 6.5 cm. in diameter and 2.5 cm. in thickness, the lamp is 10 cm. from the nearest face of the lens, while the slit is 7.5 cm. from its nearest face. The light falls on the slit as a convergent beam and is focused on the filter paper strip to give a sharp image about 1.8 cm. × 0.25 mm. as the window in the holder carrying the filter paper allows only the central 1.8 cm. of the 4 cm. wide filter paper strip to be scanned. The light passes through the paper strip and through a light filter, to be received by the photocathode of an R.C.A. 931-A multiplier photocell (cathode dimensions, 1.0 × 0.3 cm.) parallel to the slit and about 2 cm. from it. A mirror placed between the lens system and the slit deflects the beam through a right angle in order to conserve space.

Light Filters

The light filters used in this work were as follows. For amidoschwarz 10B, Ilford gelatine filters Mercury Yellow #808 (one thickness) and Red Absorbing #802 (two thicknesses) were combined with a neutral filter of density 0.5. For azocarmine B, the Ilford gelatine filter Bright Spectrum Yellow-Green #625 was combined with a neutral filter of density 0.8.

Removal of Oil

The oil was removed from the papers after scanning, using a 1:1 mixture of acetone and ether.

Planimetry

The Ott planimeter[4] was used throughout. In the wedge method, the area of the sharply defined protein-containing regions was measured.

[4] Bender and Hobein, Munich, Germany.

In the stripe method, the area of the densitometer tracing above the base line was measured.

Elution

Experiments relating surface concentration or amount of protein with optical density of the dyed protein complex have a direct relation with the requirements of the scanning technic. These experiments nevertheless can be further analyzed by adding an elution procedure. In this way it is possible to obtain the dye in free solution where there is no doubt that Beer's law is obeyed, provided a suitable light filter is used. By comparing the density of the eluted dye with the amount of protein from which it is eluted, the relationship of dye uptake with amount of protein can be tested. A colorimetric comparison of the density of eluted dye with the density of dye determined *in situ* on the filter paper relates optical density with surface concentration of the dye. After removal of the oil, the colored segments from strips prepared by the wedge method were cut out and extracted into 7 ml. of a suitable solvent, and the dye concentration estimated in a Gallenkamp photoelectric colorimeter. For amidoschwarz 10B the solvent was phenol saturated with water and containing 1% of anhydrous sodium acetate, an Ilford filter Spectrum Orange #607 being used for the estimates. For azocarmine B, the solvent

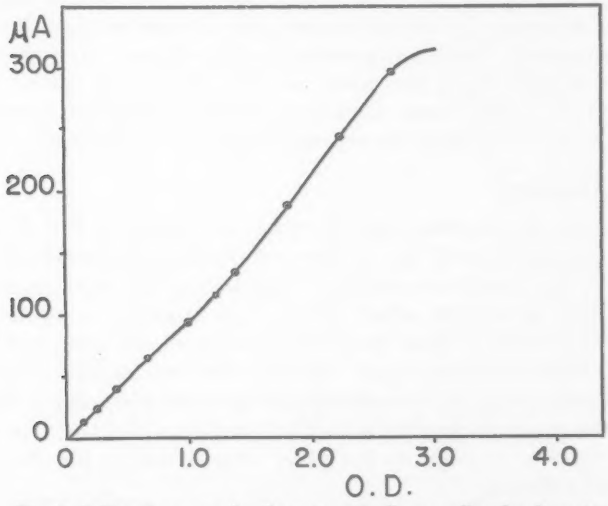

Fig. 2. Calibration curve for the automatically recording densitometer.

was 0.10N sodium hydroxide, and an Ilford filter Bright Spectrum Blue Green #623 was used.

RESULTS

A calibration curve for the recording densitometer is given in Fig. 2. This shows that between densities of 0 and 1.5, the deflection of the densitometer is linear with optical density within 1 per cent, but that above density 1.5 a curvature upwards appears. As most of the data to be given in this section will refer to the linear region of response the data have been plotted on a scale linear for microamps and the optical density scale which is on the same ordinate is therefore somewhat expanded above a density of 1.5.

Wedge Methods

Amidoschwarz Staining

Figs 3 and 4 show results from the wedge method plotted in the standard manner. Figure 3 shows results with 3 separate wedges prepared from a serum sample with an equal concentration of albumin and globulins and amidoschwarz 10B staining. The data are in accordance with Beer's law. In Fig. 4 the results from a series of 9 wedges stained with amidoschwarz are plotted and in this case the variation between strips has been removed, leaving only the variation within the strips. This has been done by plotting the best straight line for each wedge separately and then adjusting the protein concentration scales so that these lines are superimposed. The scale of protein concentration was then an average for all the wedges. Again Beer's law is confirmed up to an optical density of 1.4, but above this density there are a number of large but inconsistent departures from the law in the expected "downward" direction.

Azocarmine B Staining

The results for an azocarmine B wedge are plotted in Fig. 5. In this case all the segments of the wedge were eluted and therefore 3 corresponding sets of results are available—optical densities on paper, colorimeter readings in solution after elution, and amounts or surface concentrations of protein. Each pair of the sets has been compared and in each case there is a linear result. These results confirm Beer's law to an optical density of 1.1 for azocarmine staining and also confirm that the dye uptake is proportional to amount of protein added and that Beer's law is obeyed for the dye on the paper strip as well as for the protein estimated by dyeing.

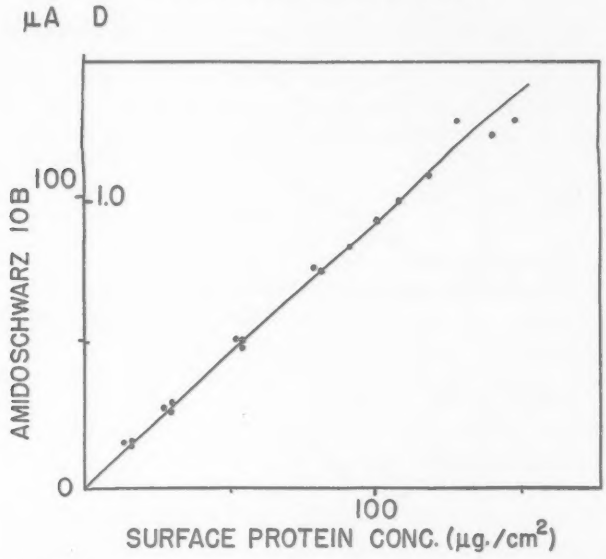

Fig. 3. Optical density related to surface protein concentration. Results with 3 wedges. (Amidoschwarz staining)

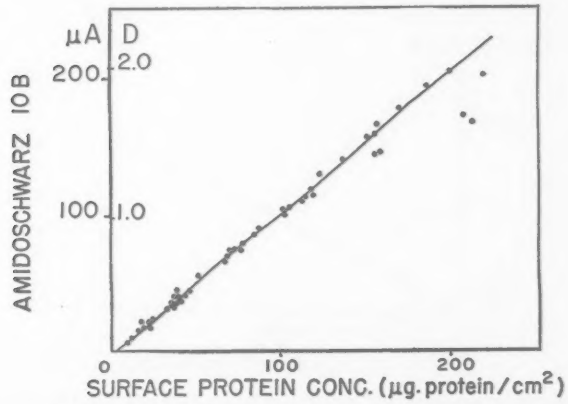

Fig. 4. Optical density related to surface protein concentration. Results with 9 wedges. The interwedge variation has been removed. (Amidoschwarz staining)

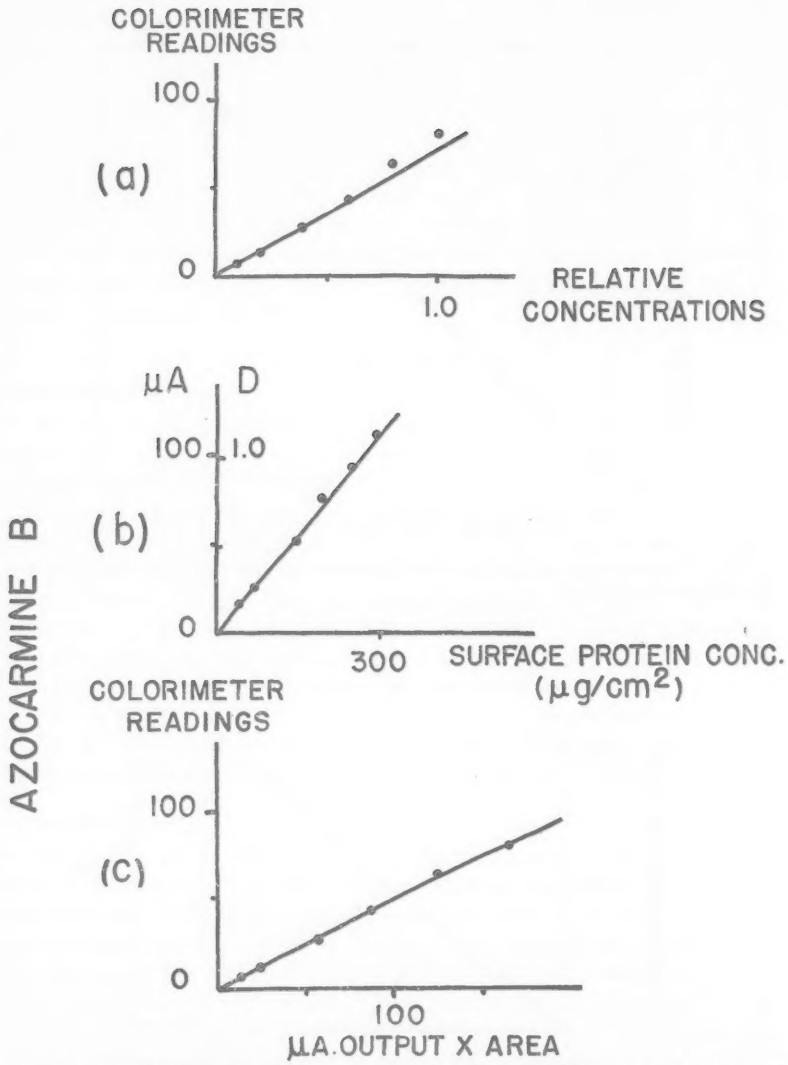

Fig. 5. Elution experiments with azocarmine B: (*a*), linearity of dye uptake with amount of protein added to the paper; (*b*), optical density related to surface protein concentration; (*c*), amount of dye eluted related to dye estimated *in situ* on the paper by densitometry.

Bovine Serum Albumin

Beer's law was also confirmed for bovine serum albumin tested for densities up to 1.0 using amidoschwarz staining, and an elution experiment was also made for amidoschwarz staining with a linear result, similar to that in Fig. 5.

Effect of Oiling and of the Light Filter

Fig. 6 shows the results with an amidoschwarz 10B wedge of omitting the oiling procedure by scanning the dry paper and of omitting the light filter. In the former case the neutral 0.5 density filter was removed and in the latter case a neutral 1.2 density filter was added in order to restore the light intensity to the usual value. If the dry paper is scanned a general increase of about 2- or 3-fold in optical density occurs due to multiple reflections in the dry paper [*cf.* Laurence (10); Price and Ashman (12)]. Also the increase is greater for low than for high densities and a curvature downward results in an overestimation of low densities. This effect has been noticed by Grassmann and Hannig (7). If the light filter is omitted, the optical density is decreased about 2-fold due to the

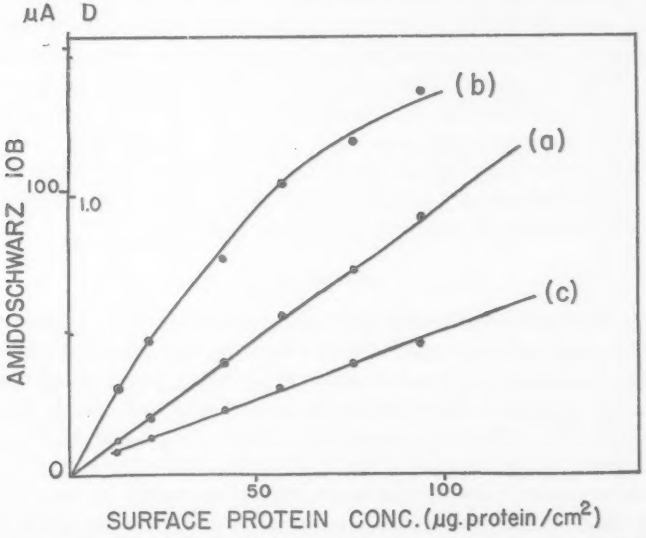

Fig. 6. Results without oiling and without a light filter: (*a*), oiled paper with light filter; (*b*), dry paper with light filter; (*c*), oiled paper with no filter. (Amidoschwarz 10B staining)

inclusion of less absorbed spectral regions of the incident light. The expected curvature downward is not apparent in this curve, which however shows a positive intercept on the density axis as found by Wenzel and Hanson (13). This latter deviation from Beer's law was not consistently obtained without a light filter and is difficult to explain.

In order to check that the light filter used was sufficient to counteract the pronounced sensitivity of the photocell to blue light, a new filter was constructed, containing three thicknesses of the Ilford Mercury Yellow 808 gelatine sheet, two thicknesses of the Red Absorbing 802, and a neutral 0.2 density filter. No differences could be detected between the results with the normal and with the new filter.

It is clear from these results that the optical density obtained for any wedge depends on the light filter and oiling procedure used, as well as on the surface protein concentration. Therefore, in order to specify the range over which linearity is found it is necessary to give details both of optical density and of protein concentration.

Results with Direct-Reading Scanner

A comparison has also been made of the results from an amidoschwarz wedge scanned by the recording scanner and by a commercial direct reading scanner with a logarithmic scale fitted with a barrier layer cell[5] but with no light filter. A linear result was obtained with either scanner, but with the commercial instrument the densities obtained were about 40 per cent less than with the recording instrument. With the commercial instrument a comparison was made between the wedges oiled as described above and "fully" oiled by removing all air in the paper immersed in the oil, using a rotary oil pump. Full oiling of the paper resulted in a further decrease of 30 per cent in the optical density.

Stripe Method

Figure 7 shows results with the stripe technic. A set of 4 protein solutions was prepared by serial 2-fold dilutions of a plasma sample. The results of scanning the four corresponding protein stripes should be areas in the ratio 8:4:2:1. By equating the largest area to 8, the deviations of the smaller areas from the expected 4, 2, and 1 are shown in the form of a histogram. There is no systematic deviation from the expected values and the scatter of the results is no more than would be expected with this technic. The peak density on the filter papers prepared by this

[5] Evans Electroselenum Ltd., Harlow, Essex, England.

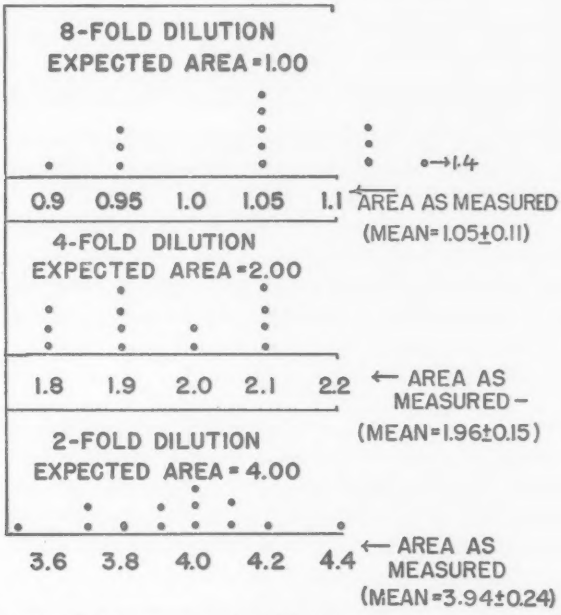

Fig. 7. Results with the stripe method. Deviations from expected "scale of two." The results shown are 13 observations for each dilution. The areas are standardized so that the area of the undiluted sample equals 8 units. (Amidoschwarz 10B staining)

method varied between 0.9 and 1.3. Although the stripe method is preferred as a test of the dynamic response of the recording system, it is a less critical test of Beer's law than the wedge method, as only a small part of the total stained area is near the peak density.

DISCUSSION AND CONCLUSIONS

The results obtained here consistently failed to show deviations from Beer's law for optical densities less than 1.4, and the use of scanning as a convenient method for differential protein estimations would appear to be justified. The methods described may enable other workers to make similar tests of the method with a minimum of preliminary development, and some may succeed in obtaining significant failure of Beer's law in their apparatus. The use of apparatus of this type would enable the variation of the deviations with arrangement of the optical system, etc., to be worked out, but the following experimental conditions are already known to lead to significant deviations:

1. Use of dry paper instead of lightly oiled paper.
2. An inadequate light filter for the photocell.
3. An illuminated slit too long to be completely covered by the protein pattern.
4. Variations in protein density over the slit, either because the slit is too wide or because the protein pattern has been applied unevenly.
5. Use of low quality filter paper containing "pin holes."
6. A dye uptake which is not proportional to protein content of the paper [Martin and Franglen (14)].

The failure of Crook, Harris, and Warren (4) to substantiate Beer's law does not indicate the most general situation for the application of the scanning method.

SUMMARY

The application of Beer's logarithmic law to the scanning of dyed protein patterns has been investigated by methods described in detail. No deviations could be found for optical densities less than 1.4 for amidoschwarz 10B or less than 1.1 for azocarmine B staining. The scanning method can be used for evaluation of protein fractions if care is taken.

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p-Toluenesulfonic Acid, Cholesterol Determination, and Explosions

Betty Jo Jones and Ferrin B. Moreland

WE HAVE BEEN USING the method of Pearson, Stern, and McGavack (1) for the determination of cholesterol for some time. It has proved to be a convenient and accurate procedure and the reaction had always gone smoothly. One day recently, however, the tubes were set up for the determination as usual, using a freshly prepared solution of *p*-toluenesulfonic acid. Solutions made from the same bottle of solid acid[1] had previously been used with smooth results. However, on this occasion, a minute or two after the serum, acetic acid, *p*-toluenesulfonic acid solution, and acetic anhydride were measured into the tubes and not mixed, a series of explosions occurred and most of the contents was blown out of the tubes. The serum blank and its zero reference tube, not containing acetic anhydride, did not blow up. A fresh solution of *p*-toluenesulfonic acid was prepared from the same solid used above, with a repeat performance of the explosions. A new solution made from a different batch of solid[2] gave smooth results. Unfortunately the offending *p*-toluenesulfonic acid was discarded before it could be analyzed to determine the change which caused the trouble. On cursory examination the bottle of solid reagent had appeared somewhat moist, particularly on the inside of the screwcap.

This note is not intended to discourage use of the Pearson, Stern, and McGavack method, which is still our method of choice for determining total cholesterol, but merely to advise caution in keeping away from above the tubes after the reagents are added, when a new solution of *p*-toluenesulfonic acid is first used. It might be well to set the rack of tubes under a shelf or to cover them with a board immediately after the components are pipeted in, when an unproved solution is used.

REFERENCE

1. Pearson, S., Stern, S., and McGavack, T. H., *Anal. Chem.* **25**, 813 (1953).

Present addresses: B. J. J., 203 Hermann Professional Bldg.; F. B. M., Biochemistry Department, Baylor University College of Medicine; Houston, Texas.

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[1] Eastman 984 (this sample had been in our laboratory for about 1 month).

[2] Matheson Pract. 2736 (this sample had been in another laboratory for over 2 years).

the Clinical Chemist

INTERNATIONAL CONGRESS ON CLINICAL CHEMISTRY

The Executive Committee of the American Association of Clinical Chemists has approved arrangements for an International Congress on Clinical Chemistry to be held in New York City during the week of September 9, 1956. The Congress will bring together eminent scientists from all over the world, in a program designed to exhibit the contributions of outstanding clinical chemists both here and abroad.

Albert E. Sobel, Chemist to the Jewish Hospital of Brooklyn and Adjunct Professor of Biochemistry at the Polytechnic Institute of Brooklyn, as well as past president of the Association, has been appointed coordinating chairman of the Congress. Committees will be announced as they are selected.

A tentative program will soon be published and will consist of symposia and contributed papers. Members of the Association and our overseas colleagues are urged to make plans to attend this Congress and participate in the scientific and social programs to be arranged.

ERNST BISCHOFF AWARD

The 1955 Ernst Bischoff Award has been presented to Michael X. Sullivan, Professor of Biochemistry at the Georgetown University in Washington, D. C. The presentation was made at the annual dinner of the Association on September 15, 1955, in Minneapolis, Minn.

SEVENTH ANNUAL MEETINGS

The Seventh Annual Meetings of the American Association of Clinical Chemists were held in Minneapolis from September 14–16, 1955. The scientific program was in participation with the Division of Biological Chemistry of the AMERICAN CHEMICAL SOCIETY, as it has been in every previous annual meeting of the Association. A report on the highlights of the meetings will appear in the next issue of CLINICAL CHEMISTRY.

REPORTS FROM THE SECTIONS

Southern California

C. Dubbs

The fourth symposium of the season was held on April 5, 1955, at the Los Angeles County Hospital. A. G. Redeker, M.D., and W. G. Bergren, Ph.D., of the University of Southern California Medical School, discussed clinical and analytical aspects of porphyrins, especially coproporphyrin, porphyrobilinogen, and the uroporphyrins. Isolation and fractionation methods were described, including paper ionophoresis and chromatography on paper and columns of alumina and Florisil.

The final meeting, held on June 7, 1955, at the Carolina Pines Restaurant, featured election of new officers. Rex Sterling, Ph.D., chemist, Los Angeles County Hospital, succeeded Dr. H. O. Carne as chairman. Kenneth D. Johnson, Ph.D., A. L. Chaney Chemical Laboratory, was elected program chairman as well as chairman-elect to succeed Dr. Sterling. Roscoe R. Schaffert, biochemist, Veterans Administration Center, Los Angeles, was elected secretary-treasurer to succeed Maxine Wertman.

As a result of the California attorney general's opinion [*Clin. Chem.* **1**, 134, (1955)] that special licensure for clinical chemists is not permitted by the California Clinical Laboratory Act, this local section has begun efforts toward obtaining corrective legislation.

Texas

The Texas Section of the American Association of Clinical Chemists is sponsoring a symposium and session of contributed papers at the American Chemical Society Southwest Regional Meeting to be held in Houston, Texas, December 1 to 3, 1955. The symposium is tentatively titled "Instrumental Methods in Clinical Chemistry". Preliminary announcement of the meeting was made in *Chemical and Engineering News*, August 15, page 3417. This ACS weekly will carry the final program. A dinner meeting is also planned as one of the events.

All clinical chemists planning to attend the meetings are requested to notify the Chairman, Dr. Ferrin B. Moreland, Department of Biochemistry, Baylor University College of Medicine, Houston, Texas.

PRINCIPLES OF LEGISLATION

The following is reprinted with permission of the AMERICAN CHEMICAL SOCIETY from the *Chemical and Engineering News* [**33**, 3105 (1955)]. It appeared as part of the Board of Directors Minutes of the AMERICAN CHEMICAL SOCIETY and embraces eight basic principles for legislation. The Committee on Clinical Chemistry of the AMERICAN CHEMICAL SOCIETY meeting in Cincinnati during April 1–2, 1955, prepared the statement. The Committee consists of Max M. Friedman, Joseph H. Gast, Rollo N. Harger, John G. Reinhold (chairman), Joseph I. Routh, Bradford N. Stanerson, Arnold G. Ware, and Hans Zinsser.

Principles of Legislation for Regulation of the Practice of Clinical Chemistry

In 1953, the ACS Committee on Clinical Chemistry presented to the Board of Directors for approval a statement of principles of legislation for regulation of the practice of clinical chemistry; no action was taken. The report was published with a request for comment.

The committee has given careful consideration to the communications received. A revised statement was presented to the Board of Directors in June 1955. Its action is given in Minute 13. In accordance with the instructions therein, the following is published:

This report has *not* been endorsed by the Board of Directors nor have the eight numbered principles for legislation been approved. It is published for the purpose of eliciting comment. Letters should be sent to John G. Reinhold, Pepper Laboratory of Clinical Medicine, University of

Pennsylvania, Philadelphia 4, Pa., with copies to the Executive Secretary of the ACS, 1155 Sixteenth St., N. W., Washington 6, D. C.

Principles of Legislation for Regulation of the Practice of Clinical Chemistry—Drafted by the Committee on Clinical Chemistry of the *American Chemical Society*.

I. The primary objective of legislation to regulate the practice of clinical chemistry is the protection of the public health.

II. Clinical chemistry is the practical application of chemical science for the purpose of providing factual data to authorized persons for use in evaluating the state of health or cause of death.

III. A license to practice clinical chemistry must be based on good character and high standards of professional competence. The competence of a clinical chemist should be judged by his ability in chemistry. He should not be compelled to qualify in other disciplines in order to practice clinical chemistry. A duly licensed clinical chemist may perform venipuncture or skin puncture for test purposes upon specific authorization from any person with authority granted under any provisions of law relating to the healing arts.

IV. Legislation to regulate the practice of clinical chemistry should require as minimal qualifications: (1) During the first five years after the act becomes effective, (a) graduation with a chemistry major from a four-year course of a college or university approved for training in chemistry by the examining board, (b) a minimum of five years of practical experience in a laboratory approved for training in clinical chemistry by the examining board. (2) Five years after the act becomes effective and thereafter, licenses should be issued only to those having (a) an earned doctorate, from a college or university approved by the examining board, in chemical science, or in biological science, medicine or public health, provided training in chemistry sufficient to establish competence is assured, and (b) one year of practical experience in a laboratory approved for training in clinical chemistry by the examining board. (3) Persons meeting the requirements of education and good character shall in addition demonstrate their competence as clinical chemists by passing an examination. (4) Persons who have directed a laboratory of clinical chemistry within two years before the date the act takes effect shall be considered eligible to practice clinical chemistry without examination.

V. Provision should be made for the supervision by licensed clinical chemists of chemical work done in laboratories where work is related to health (clinical laboratories) or for periodic evaluation of the work of such laboratories by an authorized agency such as the State Board of Health.

VI. Authorization to practice clinical chemistry shall include the right to direct a laboratory restricted to clinical chemistry. Authority to direct a clinical laboratory embracing other branches of laboratory science should be granted only to those qualified in each. However, practice should be restricted to those fields in which a scientific director has qualified.

VII. A majority of the members of a board for examining candidates and for administration of an act for the regulation of clinical chemistry should be clinical chemists. Legislation which covers several specialties related to health should recognize these in board appointments. Because of the importance of clinical chemistry among the laboratory sciences related to health, the board administering a general act should include clinical chemists.

VIII. The requirements outlined shall not apply to employees of federal, state, or municipal governments or to scientists whose work consists primarily of research.

AAAS CLINICAL CHEMISTRY SYMPOSIUM

Albert E. Sobel, Program Chairman for the Association on scientific participation in a Symposium to be held under the auspices of the subsection, the American Association of Clinical Chemists, participating in the Atlanta Meeting of the American Association for the Advancement of Science, announced the following program for the meetings.

The Symposium, entitled "Recent Concepts in Clinical Chemistry," will be held in the Dixie Ballroom of the Henry Grady Hotel, Atlanta, Ga., on Monday, December 26, 1955.

Symposium Program

Opening Remarks by the Chairman, Albert E. Sobel, of the Jewish Hospital of Brooklyn, N. Y.

Role of the Clinical Chemist in the Hospital— Joseph H. Gast, Baylor University College of Medicine, Houston, Texas

New Concepts in Acid-Base Balance —Samuel Natelson, Memorial Hospital, Rockford, Ill.

Studies on Lipid Metabolism—D. B. Zilversmit, University of Tennessee, Memphis, Tenn.

Blood Proteins—Henry G. Kunkel, Rockefeller Institute, New York, N. Y.

Meeting Program

Symposium: "Recent Concepts in Clinical Chemistry" *December 26, 1955, 2 p.m., Henry Grady Hotel, Dixie Ballroom.*

AACC Dinner: *December 26, 6 p.m., Henry Grady Hotel, Dixie Ballroom.*

Contributed Papers: *December 27, 9 a.m., Henry Grady Hotel, Room B.*

Contributed Papers: *December 27, 2 p.m., Henry Grady Hotel, Room B.*

LECTURING ABROAD

Harry Sobotka, Mount Sinai Hospital, New York City, is visiting the Near East during September and October. He will give a number of lectures and hold conferences on subjects of physical biochemistry at the University of Athens and the National Technical University in Athens, Greece, at the University of Ankara and Istanbul, Turkey, and at the University of Jerusalem and at the Weizmann Institute in Rehovot, Israel.

NEW CATALOGUE SUPPLEMENT

The Arthur H. Thomas Company, Philadelphia, Pa., announces publication of a 264-page supplement to their 1472-page general catalogue. The Supplement follows the tradition of the catalogue and provides factual, detailed, and up-to-date listings of items added to their stock since publication of the catalogue in 1950. Also included is a price list covering all current items in both catalogue and supplement.

ERRATUM

In the article, "A Note on Quantitative Urobilinogen Determinations", by Bernard Balikov, Vol. 1, 264 (August 1955), the abscissa label of Fig. 1, page 265, should read "2% glacial acetic acid assumed to yield 100% recovery."

ABSTRACTS

Editor: Ellenmae Viergiver. *Contributors:* Joseph A. Annino, Gladys J. Downey, Clyde A. Dubbs, Alex Kaplan, Margaret M. Kaser, Miriam Reiner, Heraert Thompson

Uptake of radioactive iodine in the thyroid of patients with impaired liver function. R. Mueller, C. C. Brausch, E. A. Hirsch, R. S. Benua, and B. M. Dobyns. (*Western Reserve University School of Medicine, Cleveland, Ohio*)

The I^{131} thyroidal uptake was 50 per cent or higher in 20 of 50 patients with liver disease without evidence of thyroid disease. Among the patients with liver disease, elevated I^{131} thyroidal uptakes were associated most frequently with spider angiomata, jaundice, and microscopic evidence of severe cellular liver damage. No correlation could be established between elevated I^{131} thyroidal uptakes and either the presence of ascites or the results of any single test for liver function.

In spite of the elevated I^{131} uptakes by the thyroid in some patients with liver disease, there was no evidence of increased production of thyroid hormone, either by clinical findings or by the basal metabolic rate, the serum protein-bound iodine level, or by the height of thyroid cells. Although abnormal kidney function may be present in patients with cirrhosis, the normal urinary excretion of radioiodine found in sample patients in this series indicates that renal disturbance at the time of the tests was not the case of the elevated I^{131} thyroidal uptakes.—*J. Clin. Endocrinol. & Metab.* **14,** 1287 (1954).

M. R.

The influence of single doses of propylthiouracil on the kinetics of iodine accumulation by the thyroid glands of normal and hyperthyroid subjects. S. H. Ingbar (*Walter Reed Army Medical Center, Washington, D. C.*)

An evaluation was made of the influence of propylthiouracil in single doses of 25, 50 and 100 mg. on the intrathyroidal mechanisms of hormone formation in 10 euthyroid and 8 hyperthyroid patients. No reduction of the ability of the gland to concentrate inorganic iodide was observed. A diminution in the thyroidal rate of transfer of iodide from the plasma into the glandular hormone pool occurred in all patients as the result of an inhibition of the rate of protein binding of thyroidal iodide. The magnitude of the inhibitory effect was found to be proportional to the control level of the protein-binding rate and to the dose of antithyroid agent administered.—*J. Clin. Endocrinol. and Metabolism* **15,** 331 (1955).

(M. R.)

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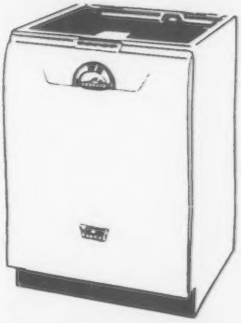

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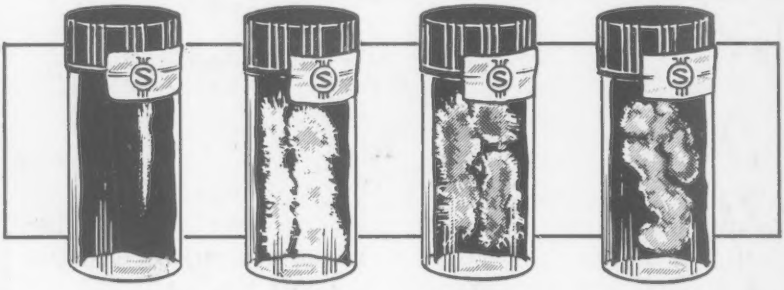

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